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Review

Application of tandem mass spectrometry for the analysis of long-chain carboxylic acids

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Abstract

The application of MS–MS for the analysis of long-chain carboxylic acids and their esters has proved enormously successful but expensive. It is discussed mainly on basis of results obtained with different instruments with lesser attention to principles of the method, which have been adequately reviewed elsewhere. The use of electrospray ionization (ESI) has greatly increased the sensitivity of the method and has permitted assay of total lipid extracts. The combination of HPLC with electrospray and single quadrupole mass spectrometry, LC–ESI-CID-MS, rivals the triple quadrupole MS–MS application in many instances at considerably lower cost. However, LC–ESI-MS–MS remains the most desirable system at the present time for lipid ester analyses.

Contents

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List of abbreviations

CI	Chemical ionization
CID	Collision-induced dissociation
CF	Continuous flow
CoA	Coenzyme A
dc	Direct current
DCI	Desorption chemical ionization
DG	Diacylglycerol
EI	Electron impact ionization
ES	Electrospray
FAB	Fast atom bombardment
FD	Field desorption
GC	Gas chromatography
GPC	Glycerophosphocholine
GPE	Glycerophosphoethanolamine
GPL	Glycerophospholipid
GPS	Glycerophosphoserine
HEA	Hydroxyeicosanoic acid
HETE	Hydroxyeicosatrienoic acid
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
LSI	Liquid secondary ionization
LT	Leukotriene
LTC	Leukotriene C
LTD	Leukotriene D
M	Molecular ion
MG	Monoacylglycerol
MIKES	Mass analyzed kinetic energy spectra
MS	Mass spectrometry
MW	Molecular mass
NBA	Nitrobenzyl alcohol
NICI	Negative-ion chemical ionization
P	Parent ion
PA	Phosphatidic acid
PAF	Platelet activating factor
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEt	Phosphatidylethanol
PFB	Pentafluorobenzyl
PG	Phosphatidylglycerol
PGE	Prostaglandin E
PI	Phosphatidyl inositol
PLT	Peptide leukotriene
PS	Phosphatidyl serine
QqQ	Triple quadrupole mass spectrometer
rf	Radio-frequency
SFC	Supercritical fluid chromatography
SIM	Selected ion monitoring
SPH	Sphingomyelin
TG	Triacylglycerol
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
TPPV	Triphenylphosphonium valeryl amide
TS	Thermospray

1. Introduction

Tandem mass spectrometry or mass spectrometry–mass spectrometry (MS–MS) is now an established method for the structural analysis of long-chain carboxylic acids. It not only provides additional information but can also improve selectivity and sensitivity. It is achieved by arranging two or more mass spectrometers in a series. Specificity and sensitivity is further improved by coupling MS–MS to capillary gas chromatography (GC) and high-performance liquid chromatography (HPLC). This development has been closely related to instrumental innovation, advances in sample introduction techniques, and ionization methods, and to advances in fast computation. The variety of analytical problems solved by means of MS–MS is large and the number of examples in the lipid area is gaining rapidly. Only applications to analysis of long-chain fatty acids and their esters are described here, with illustrations selected from the biomedical area. Detailed fundamental reviews [1] and applications in other areas [2] are found elsewhere. Selected applications of MS–MS in the structural analyses of triacylglycerols

[3], glycerophospholipids [4], sphingolipids [5] and eicosanoids [6] have appeared recently.

2. Principles of MS–MS

In principle MS–MS is mass spectrometry of a specific ion selected from the initial mass spectrum. The selected parent ion is allowed to enter a field-free reaction region, where it may undergo unimolecular or collision-induced dissociation (CID). The resulting fragments (daughter ions) are analyzed by a second mass spectrometer, which provides the MS–MS spectrum. Although commonly referred to as MS–MS, tandem mass spectrometric analysis is usually MS–CID-MS [7]. Generally the intermediate field-free region is used as a collision chamber where the internal energy of the parent ion is increased due to collisions with some target gas. This increased internal energy induces the ion to fragment into daughter ions, which are then analyzed in the second mass analyzer. The daughter ions are characteristic of the structure of the parent ion and the MS–MS allows the determination of that structure [1,8]. The maximum amount of the original kinetic energy of the parent ion that can be transformed into the internal energy needed for CID is regulated by the need to conserve the total momentum and is given by Eq. 1:

$$E_{max} = M_2/(M_1 + M_2) \cdot E_{ion} \quad (1)$$

where E_{max} is the maximum amount of internal energy acquired in the collision, E_{ion} is the original kinetic energy of the parent ion, M_2 is the mass of the target atom or molecule, and M_1 is the mass of the parent ion.

Although the first mass spectrometer separates the mixture as does the gas or the liquid chromatograph and the second determines the mass spectra of the molecular ion, the method differs from both GC–MS and LC–MS, which are based on chemical separation. In MS–MS the separation is based on physical properties. This approach allows assessment of neutral fragment loss and parent ion scans, which have no counterpart in GC–MS or LC–MS. In addition, in MS–MS, the first mass spectrometer serves to filter out the background and improves signal-to-noise ratio.

3. Instrumentation

MS–MS has become a convenient and often indispensable tool for lipid structure analysis. The instrumentation ranges from simple ion trap arrangements to high-resolution double-focusing machines with supplementary sectors of varying ionization potential. All of them can be combined with gas and liquid chromatographs, which provide preliminary resolution of analytes. However, with the increased complexity of the instrumentation comes increased cost, increased down time and decreased overall reliability of the operation. Change-over from one mode of operation to another is cumbersome, frequently requiring the assistance of a factory-trained engineer. Therefore, instruments and operators dedicated to specific applications constitute the most efficient but also the most expensive way of using the instruments.

3.1. Hardware

The basic hardware employed in MS–MS of lipids has been recently discussed by Le Quere [4]. This section summarizes and updates the earlier review. Originally, MS–MS was done on two-sector, double-focussing instruments composed of an electric (E) and magnetic (B) sector arranged in forward (EB) or reverse (BE) geometry. Scans of daughter ions were obtained using the BE instrument, where the parent ion selected with the magnet is dissociated in a collision cell located in the intersector region. The fragment ions were analyzed by scanning the electric sector voltage. More recently, instruments have been developed where one or two analyzers are added to double-focussing mass spectrometers. The reason for adding one sector to a double-focussing instrument is to provide high mass resolution for the first stage of MS–MS experiments, so that isobaric parent ions can then be resolved. Another advantage is the introduction of a third field-free reaction region. It allows

examination of consecutive reactions in MS–MS–MS experiments, where isomeric parent ions can be distinguished.

The most widely used are the triple quadrupole instruments (QqQ), in which the first and third quadrupoles are operated in the usual way for mass analysis by combination of radio-frequency (rf) and direct current (dc) voltages. The second quadrupole is operated in the rf-only mode. The purpose of the second quadrupole is to serve as a high-pressure collision cell and to focus the fragment ions scattered from the central axis of the ion beam by the collision process. The major advantage of the QqQ instrument is its operational simplicity for MS–MS measurements. Quadrupoles in general, have rapid scanning capabilities, unit mass resolution, small size, simplicity of operation under full computer control and a relatively low cost. The main disadvantages are limited mass range (1000 or 2000) and lack of high resolution.

In order to take advantage of the best features of both analyzers, hybrid instruments have been developed, in which sectors and quadrupoles are combined in single MS–MS instruments. The most common hybrid instruments are those in which a double-focussing sector instrument precedes a qQ system, with the first q being an rf-only gas collision cell (EBqQ or BEqQ). The cost of such instruments is much lower than that of four-sector instruments and allow high-resolution parent ion selection and unit mass resolution of daughter ions.

Analytical evaluation of complex mixtures of natural lipid esters demands a prior chromatographic separation by GC or HPLC in conjunction with mass spectrometric identification. The use of short GC columns has been shown [9] to be advantageous for the separation of a wide range of compound classes from fatty acids to mono-, di- and triacylglycerols. These columns offer the potential for reduced thermal degradation, lower temperature of operation and faster analysis time, as well as higher sensitivity due to sharper peaks and higher sample concentrations. Likewise, the use of reversed-phase HPLC [3,10] may be advantageously combined with MS–MS via special interfaces. In case of natural triacylglycerols, two or more chromatographic techniques may have to be combined to obtain a triacylglycerol mixture simple enough for analysis even by MS–MS [3].

3.2. Methods of scanning

The various combinations of sectors and quadrupoles allow different modes of scanning, some of which are better suited for MS–MS of lipids than others. For more detail the reader should consult Le Quere [4], who has recently reviewed the main methods. Double-focusing instruments of reverse geometry allow parent–daughter ion relationships to be determined by acquisition of mass-analyzed kinetic energy spectra (MIKES). In this arrangement, a precursor ion that is selected in the magnetic sector dissociates in the collision cell, located in the field-free region between the magnetic and the electric sectors. The electric sector then filters the resultant ions according to their kinetic energy. This ion kinetic energy spectrum can then be converted to a mass-analyzed daughter ion spectrum because a linear relationship exists between the electric field strength necessary to pass through the analyzer and the daughter ion mass.

More complex scans are needed to obtain other parent–daughter ion relationships in the reverse geometry instruments. When the collision cell is located in the first field-free region of instruments of either conventional or reverse geometry, other MS–MS scans are possible. Covariant scans, in which two of the three values (accelerating field, V, electric field, E, and magnetic field, B) are changed but always retain a specified mathematical relationship [8], can also provide daughter–parent ion relationships. These scans are called "linked scans".

In the linked B/E scan, B and E are scanned simultaneously in such a way that the ratio B/E is kept constant. In this case only ions of the same velocity can pass through both sectors, and daughter ions of a given parent ion can reach the detector. This method of scanning is widely used in the lipid field. B^2/E scan leads to a parent ion MS–MS spectrum. All ions that dissociate to chosen daughter ions in the first field-free region

of a two-sector instrument of either geometry are detected. The $B^2(1-E)/E^2$ scan allows the detection of all parent ions that lose a constant neutral fragment in the first field-free region. This method of scanning has proven useful in analysis of complex mixtures as it allows one to choose only those ions in the original spectrum that belong to a particular class of molecules.

In QqQ instruments, a daughter ion scan is obtained simply by setting the first Q to pass the parent ion and scanning the last Q. Conversely a parent ion scan is obtained by setting the last Q for the mass of the chosen daughter ion, and scanning the first Q. A neutral fragment loss scan is obtained by scanning both mass analyzers in concert at the same rate, but with a mass offset equal to the selected neutral loss. This makes QqQ the instrument of choice for extensive GC–MS–MS. It does not, however, allow high-energy CID nor MS–MS–MS experiments to be performed.

In ion trap instruments the main feature is the separation of the MS–MS analysis in time, rather than in space as in conventional MS–MS instruments. The sequence for an MS–MS experiment using an ion trap includes ionization, rejection of all ions except the parent ion, dissociation of the parent ion and trapping followed by ejection of the daughter ions [11]. MS–MS is achieved by accelerating the parent ion by applying a supplementary rf voltage (resonant excitation) across the end caps. The increased kinetic energy causes fragmentation of the parent ion due to more energetic collisions with the buffer gas normally present in the ion trap.

Hybrid instruments use more complicated scan routines in order to take advantage of all the reaction regions operating at low or high collision energy.

3.3. Methods of ionization

The soft ionization MS techniques, such as fast atom bombardment (FAB), field desorption (FD), thermospray (TS) and electrospray (ES) have the ability to ionize lipid molecules without causing extensive fragmentation. This generally leads to mass spectra characterized by molecular adduct ions. Ionspray, which can be used with flow-rates compatible with HPLC is similar to electrospray except that the initial nebulization process is assisted by pneumatic or ultrasonic means. ES favours multiply charged ions for proteins but fatty acids and their esters usually acquire only one charge per molecule.

The desorption-ionization methods have been most often employed in the past to generate the parent ions of large polar organic molecules. More recently, TS and ES have also been used for this purpose because they may be coupled to HPLC by appropriate interfacing. The most common method used to obtain fragmentation of the molecular ionic species produced by a "soft ionization" process is CID. It should be noted that in sector MS–MS instruments the incident ion kinetic energy is usually between 6 and 8 kV, whereas in quadrupole MS–MS instruments it is usually in the vicinity of 20–40 eV. In the latter case the energy available for fragmentation may be increased by multiple collisions brought about by increased cell pressure.

3.4. Pseudo MS–MS

ES has a very unique property that distinguishes it from other soft ionization techniques. It can be used to produce CID spectra of singly charged species with greater sensitivity than can be achieved with QqQ ES-MS–MS systems. This permits experiments not practical with other ionization methods. Fig. 1 illustrates how an LC–ES-CID-MS can be used in place of ES-MS–MS [7]. The ionization in this instance was effected with the Hewlett-Packard dual-stage ES ionization source. When analytes are present in the sprayed solution, molecular adducts, typically protonated parent ions $[M+H]^+$, are formed and guided into the reaction region of the mass spectrometer between the exit of the glass capillary and the first skimmer. If a low voltage of 50–120 V is applied to the capillary exit, the molecular ionic species remain intact and the molecular mass of the analyte is obtained. However, if higher voltages are applied to the capillary exit, extensive and reproducible fragmentation of the molecular adduct ion can be effected.

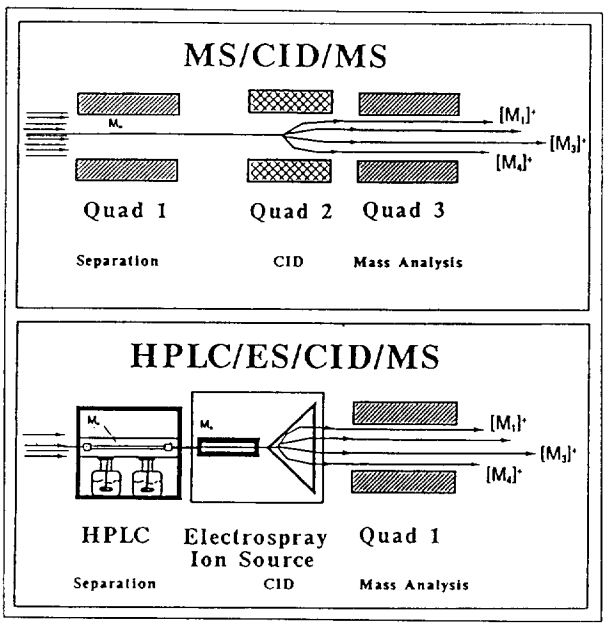

Fig. 1. Schematic drawing comparing an MS–CID-MS triple quadrupole system (upper panel) with an HPLC–ES-CID-MS single quadrupole system (lower panel). Reproduced from Ref. [7] with permission.

The fragmentation arises by means of a CID process, which is identical to that, which takes place in the collision chamber of a QqQ MS–MS system. However, the "product ions" or fragment ions produced in the ES-CID region are more effectively transferred into the quadrupole mass analyzer with much less off-axis scatter than the transfer of fragment ions from an MS–MS collision process in a QqQ system [12]. The more effective ion transfer is due to the location of the collision process within the ES lens system. It takes place in a "guest zone", which has been generated by the supersonic expansion of the nitrogen gas exiting the capillary. Thus, for LC–ES-CID-MS, an HPLC system is used instead of a mass analyzer to carry out the separation of a single component (Mn) in the mixture. The ES source is used as the "collision chamber" where CID takes place and finally, a single quadrupole (Quad 1) is used to mass analyze the "product" ions.

In most experiments, HPLC is a better way for separation than the use of a mass filter, particularly for isomeric compounds such as positional isomers, *cis/trans* isomers, *syn/anti* isomers, diastereomers and enantiomeric compounds, where MS–MS spectra are not sufficiently different to distinguish their individual identities. Prior chromatographic separation is also important when a charge suppression compromises the sensitivity of detection of components. For the vast majority of analytical problems, an HPLC separation is significantly more useful and less expensive than the use of a mass spectrometer for the "mass separation". It has been concluded [12] that the claim that MS–MS has greater specificity than single MS does not apply when comparing ES-MS–MS to LC–ES-CID-MS.

It is also known from previous work that the overall transmission of ions following two mass filtering steps such as in MS–MS, generally reduces the ion yield to the detector [13]. The latter reports 6% ion yield at mass m/z 167 and less than 1% ion yield at m/z 2000 [13]. There is a loss of 20–100 times in sensitivity when compared to single mass filtering. While the above use may hold true for a limited number of cases, the general use for MS–MS is a less cost-effective combination of tools than is the LC single Q system consisting of the LC–ES-CID-MS combination. For sensitivity, the advantage of an HPLC for compound separation becomes even more significant when the "soft ionization" technique being used is ES. When using ES analysis, a charge suppression effect may have an important impact on sensitivity and reproducibility for components in complex mixtures.

The best results in terms of sensitivity, specificity and reproducibility are obtained by using HPLC as a separation device combined with the ES–CID-MS method instead of relying on the selection of specific masses from mixtures and using the first Q of a QqQ system for the separation. Voyksner and Pack [12] have shown that CID daughter ion yield to the detector for a single-Q system is nearly 90% as compared to only a few percent for the QqQ system. Spectral reproducibility due to the inherent charge of the ES ion source and its supersonic quiet zone, yield a highly reproducible daughter ion spectrum from the single Q CID process. Voyksner and

Pack [12] found single quadrupole CID variation to be less than ± 10% reproducibility for antibiotics and pesticides analyzed in their laboratory.

The highly reproducible daughter ions obtained using a single Q combined with CID has caused some investigators to suggest that MS spectral libraries are now possible [12]. Additionally, the daughter ions may be used for quantitation by means of internal standards and selecting the ions of interest and varying the internal standard to the analyte of interest.

4. Applications

Natural lipids contain fatty acids differing in chain lengths, degree of unsaturation and substitution of the hydrocarbon chain. The determination of the location of double bonds in carbon chains and the position of a substitution has been a demanding problem and many reviews have appeared recently [14–16]. However, there is no universal method for determining the structural modifications of the fatty acids. The determination of molecular association and positional distribution of fatty acids in glycerolipid molecules has presented a further challenge. Both problems have aroused the interest of users of tandem mass spectrometry.

4.1. Fatty acids and alcohols

Fatty acid mass spectrometry dates back to the earliest explorations of the ion chemistry of lipid molecules. The location of double bonds and methyl and hydroxyl substituents was initially studied by means of special derivatives and GC–MS with electron impact ionization [14,16]. More recently, the softer ionization techniques (FAB, ES) have permitted the study of these derivatives as well as underivatized fatty acid molecules by (MS–CID-MS) [15].

4.1.1. Saturated and branched-chain compounds

While the EI spectra of the fatty acid methyl esters do not provide structural information [14], the picolinyl esters [15] yield sufficient data for the location of the branch points in mono- and polymethylated fatty acids.

It has been shown that CID of FAB-derived carboxylate anions of non-derivatized fatty acids yields information which allows the substance to be identified as a fatty acid and modifications such as branching and double bonds can be located [17]. Jensen and Gross [18] have shown that MS–MS in combination with FAB is especially useful for the determination of the iso- and anteiso-fatty acids. Mass spectra were obtained with an EBE-type triple analyzer tandem mass spectrometer and CID with helium was allowed to take place in a collision chamber between the second and third sector. Negative-ion spectra were obtained and CID spectra of the $[M-H]^-$ ions were used to distinguish between the isomers. The fragmentation was noted to take place by a charge-remote mechanism. An acid with anteiso-fatty acid branching could be readily distinguished from one containing iso-branching by ions resulting from the loss of the elements of CH_4 and C_2H_6 but not C_3H_8 from the former. The 16-methyl heptadecanoic, 18-methyl nonadecanoic, 14-methyl hexadecanoic and 16-methyl octadecanoic acids analyzed individually gave good results but a mixture of isomeric fatty acids posed a potential problem. Nevertheless, the general distribution of the abundances of ions was so reproducible that the presence of both eicosanoic and 18-methyl nonadecanoic (both of m/z 311) could be recognized from the CID spectrum of the $[M-H]^-$ (m/z 311) of a 1:1 mixture of the two acids.

Deterding and Gross [19,20] have shown that CID daughter ion spectra of the $[M+H]^+$ ion of the picolinyl derivatives show many of the same features as those present in the EI spectra. They note that these derivatives tend to fragment by a charge-remote mechanism, in contrast with the CID of the $[M+H]^+$ ions of the fatty acids and their methyl esters, where carbocation intermediates are formed by charge-mediated processes [19]. The isomerization of carbocations causes loss of structural information by charge-mediated processes leading to loss of water and methanol from $[M+H]^+$ ions of acids and methyl esters, respectively.

MS–MS spectra also reveal the position of each methyl group in the spectra of the picolinyl ester of phytanic (3,7,11,15-tetramethylhexadecanoic) acid [19]. There was a nearly total suppression of the fragments that involve the losses of the alkyl and methyl branches.

From the CID spectrum of trans-9,10-methyleneoctadecenoic acid it was possible to find the position of the cyclopropane ring, but the location was not as readily determined from the general appearance of the spectrum as were other substituents [19].

The FAB-MS–MS method is also useful for identifying short-chain fatty acids. Hunt et al. [21] used MS–MS for the identification of short-chain carboxylic acids found in metabolic abnormalities. Upon CID, charge-remote fragmentations were still observed for picolinyl esters of low molecular mass saturated acids such as *n*-valeric and heptanoic acids.

4.1.2. Unsaturated compounds

One of the difficult problems in determining the structure of unsaturated fatty acids is suppression of double-bond migration generally observed in the EI mass spectra. A partial solution to the problem is derivatization at double-bond positions and functional group derivatization [14,15]. In an early MS–MS study, the double bonds were derivatized into amino alcohols via epoxy methyl esters [22]. MIKES-CID spectra of the pseudomolecular ions formed by ammonia ionization showed intense signals allowing the location of the double bond in the monounsaturated fatty acids.

Another approach is provided by the charge-remote fragmentation [17]. This type of fragmentation is observable in CID spectra of certain closed-shell ions that are mostly favoured under conditions of FAB or liquid secondary ion (LSI) mass spectrometry and the fragmentations provide structural information on fatty acids containing intact double bonds [23]. Upon FAB ionization, fatty acids can be desorbed as very stable carboxylate anions $[M-H]^-$ or dilithiated cations $[M-H+2Li]^+$ [24]. Subsequent CID causes charge-remote fragmentations, which, with a saturated precursor, lead to a series of fragments by losses of C_nH_{2n+2} units from the alkyl chain. The resulting CID spectrum contains a series of product ions regularly spaced by intervals of 14 mass units. An unsaturation suppresses cleavages of the double bond and at the vinylic positions and shifts the masses of certain product ions. These unique features allow for the unequivocal diagnosis of the fatty acid's connectivity [24]. Subsequently, Adams and Gross [23] have extended the chemistry and have evaluated its analytical usefulness for determining double-bond location in an extensive series of unsaturated fatty acids. The alkali-metal cationization was particularly useful for the analysis of polyunsaturated fatty acids with four or more double bonds [23]. The detailed mechanism of charge-remote fragmentation remains unclear. The original reaction pathway postulated by Jensen et al. [24] was based on labelling data and involved a concerted, symmetry-allowed 1,4-H_2 elimination with concomitant formation of alkenes and terminally unsaturated fatty acid ions. However, mechanistic inconsistencies have been reported for monounsaturated fatty acids [25,26]. Furthermore, the co-existence of a secondary but nonetheless significant fragmentation pattern originating from simple bond cleavages has been described by Bambagiotti et al. [27,28] for the CID fragmentation of fatty acid carboxylate ions formed upon dissociative electron capture of fatty acid methyl esters. In addition, Vysocki and Ross [29] have shown for a number of long-chain functionalized alkanes and alkenes that radical ions, whose formation involves a homolytic C–C cleavage, have an enhanced relative abundance when applying a low energy CID, and have suggested that charge-remote fragmentations are initiated by homolytic C–C cleavages. Contado et al. [25] have confirmed that unsaturated fatty acid ions can undergo losses of radicals by cleavages at the allylic bonds.

Cordero and Wesdemiotis [30] have investigated the elimination of the neutrals upon high-energy CAD of FAB-generated fatty acid ions. The fatty acids studied included the saturated C_{11}, C_{14}, C_{16}, C_{18}, as well as the monounsaturated C_{11} and C_{16} acids. The experiments were performed on an EBE-type MS–MS instrument

and the fatty acids were ionized by FAB. $[M-H]^-$ or $[M-H+2Li]^+$ precursor ions were mass-selected and subjected to CID by collision with helium in a cell between the last two sectors. The CID spectra of $[M-H]^-$ from monounsaturated fatty acids have been observed to present a discontinuity or "gap" in their fragment ion abundance pattern.

Claeys et al. [31] have used this methodology to determine the double-bond position in vaccenic acid and in long-chain 6-alkenyl salicylic acids, which are non-isoprenoid phenolic acids. The vaccenic acid and vaccenic alcohol are known to contain a double bond in the ω-7 position. The product ion spectra showed the expected pattern for ions formed by charge-remote fragmentations in the alkenyl chain as reported first by Tomer et al. [32] for $[M-H]^-$ precursor ions and later for $[M-H+2Li]^+$ cations of fatty acids and $[M+Li]^+$ cations of fatty alcohols. The charge-remote fragmentations involving allylic cleavage of the C_{13}–C_{14} bond and homolytic cleavage of the C_{15}–C_{16} bond yielded the most abundant product ions. It could also be clearly seen that ions corresponding to a remote charge fragmentation had the lowest abundance and involved cleavage of the C_{11}–C_{12} bond, which can only occur after isomerization.

Claeys et al. [31] have examined higher homologues of the anacardic acid, 6-[8′(Z)-pentadecyl]salicylic acid, which contain C_{17}, C_{19}, and C_{21} alkenyl chains and are found in leaves and twigs of *Spondias mombin* used in African traditional medicine for various purposes, but could not be isolated in sufficient amounts for ozonization. Well known products of this group include the anacardic acids which have a C_{15} chain containing 0, 1, 2 or 3 *cis*-double bonds, which are separated by methylene groups. The anacardic acids have molluscicidal properties. Claeys et al. [31] applied caesium FAB in combination with CID and linked scanning at constant B/E to obtain product ion spectra of the intense $[M-H+2Li]^+$ and $[M-H]^-$ ions.

The CID product ion spectra obtained for $[M-H+2Li]^+$ precursor ions of the 6-alkenyl salicylic acids showed specific patterns typical of ω-7 unsaturation formed by charge-remote fragmentations, although the patterns were less clear than those obtained in case of the vaccenic acid or vaccenic alcohol. Two discontinuities within the series of homologous product ions reveal the position of the double bond: a dip in the relative abundances and a shift by two mass units. The mass shift appears to be the more reliable indicator. The authors point out that the interpretation of $[M-H+2Li]^+$ product ion spectra is straightforward and less complex than the analysis of $[M-H]^-$ product ion spectra. In order to explain the formation of the radical cations and terminally unsaturated ions, which are found for the spontaneous and CID fragmentation of lithium-cationized 6-alkenyl salicylic acids, Claeys and VandenHeuvel [26] have proposed radical mechanisms which involve a homolytic C–H cleavage. This mechanism is consistent with the charge-remote concept and also explains the observed enhanced abundances of ions whose formation involves formal benzylic, homobenzylic and homoallylic cleavages. The enhanced abundance of ions whose formation involves formal allylic cleavages were rationalized by resonance stabilization of intermediate allylic radical ions. On the basis of the identification of ω-7 and ω-6 unsaturated long-chain alkenyl salicylic acids it was suggested that ω-7 and ω-6 monounsaturated fatty acids serve as biogenetic precursors.

A similar strategy has also been proposed as a method of locating double bonds in unsaturated fatty alcohols [33]. Fatty alcohol $[M+Li]^+$ ions were shown to decompose analogously to fatty acid $[M-H]^-$ anions. The mechanism was consistent with losses of neutral alkenes and H_2 to give terminally unsaturated lithiated alcohols [17]. The remote decomposition of modified alcohols clearly indicated hydrocarbon chain length and location of double bonds [33]. In contrast the fragmentation of fatty alcohol $[M+H]^+$ ions does not result in structurally informative CID spectra [17]. The detection limit for lithiated fatty alcohols is much higher than that for fatty acids, and the applicability of the method to fatty alcohols may be limited.

Nakamura et al. [34] have reported a novel variation of charge-remote methodology for

locating double bonds in fatty alcohols of submicrogram quantity. The method involves charge localization at the hydroxyl group by microderivatization and is applicable to a wide range of compounds, including monounsaturated and polyunsaturated fatty alcohols. For this purpose, the sulfate ester was selected as the charge-localized derivative and the method of Hoiberg and Mumma [35] was used for the derivatization of fatty alcohols. The carboxyl derivative was not used because CID of the carboxylate ion involves considerable competitive reactions [23,24,36]. The other negative charge carrier candidates that can be easily derived from alcohols are phosphate esters [37] and sulfate esters [38]. Primary ions produced by LSI were given 4 keV energy and fragmented in a He collision chamber [34]. All spectra were obtained with a four-sector EBEB-MS–MS instrument. The ions attributable to the sulfate were predominant in the spectra and there was no evidence suggesting the presence of any side reactions. Since intact fatty alcohol molecules do not have any preformed negative charges, they should give only weak peaks in negative-ion spectra even if they remained in reaction mixtures.

Generally, high-energy CID product spectra of sulfate anions show clear charge-remote fragmentation patterns [38] and for oleoyl alcohol clearly shows the double-bond position in the carbon chain. Although the fragmentation pattern of the sulfate was closely related to that of oleic acid, the product ion yield from the sulfate anion was a factor of 2 or 3 higher than that from the carboxylate anion. The present method was sensitive enough to analyze submicrogram-quantity samples [34]. The detection limit for double-bond location in oleoyl alcohol by the present method was estimated to be 40 ng or less and is comparable to that for lithiated fatty acids. Double-bond positions in polyunsaturated alcohols can also be analyzed by the sulfate method. Fig. 2 shows the CID product ion spectrum of arachidonyl sulfate prepared from 1 μg of the corresponding alcohol. The spectrum shows a simple charge-remote fragmentation and is easily interpreted to yield the position of the double bonds.

4.1.3. Hydroxy and epoxy compounds

Unsaturated fatty acids can be oxygenated to hydroperoxy compounds by lipoxygenation or peroxidation and then reduced to hydroxy compounds by peroxidase activity while others undergo transformation to epoxy hydroxy derivatives or hepoxilins [39]. Many hydroxy-substituted unsaturated fatty acids possess potent biological activities. The structure of these compounds is typically determined by GC–MS after multistep derivatization procedures including methylation, hydrogenation and trimethylsilylation [14,16]. The controlled fragmentation of the substituted fatty chain by EI of the picolinyl derivatives has provided another successful approach [15].

As in the case of regular fatty acids negative-ion formation by FAB of underivatized hydroxy fatty acids results in abundant carboxylate anions, and CID-MS–MS of these ions produces structurally significant products [40–42]. Recently, Deterding et al. [43] has described the high-energy CID spectra of several hydroxy-substituted unsaturated fatty acids and Wheelan et al. [44] have applied continuous flow injection FAB-MS–MS analysis to underivatized monohydroxy-substituted fatty acids and the abundant carboxylate ions were studied using low-energy CID with QqQ mass spectrometry. Even though low-energy CID does not provide structurally useful fragments for hydroxy-substituted saturated fatty acids it does for substituted unsaturated fatty acids. Wheelan et al. [44] have reported the CID spectra of monohydroxy-unsaturated fatty acids derived from oleic, linoleic, linolenic and arachidonic acids. The spectra revealed structurally informative ions as to the position of the hydroxyl substituent in relationship to the sites of unsaturation. Supported by evidence from the spectra of deuterium-labelled analogs five mechanisms were proposed for the fragmentation of hydroxy-substituted unsaturated fatty acids which are dependent upon the presence of α- or β-unsaturation sites. These mechanisms include charge-remote allylic fragmentation, charge-remote vinylic fragmentation, charge-driven allylic fragmentation, charge-driven vinylic fragmentation, and homolytic fragmentation by an oxy-

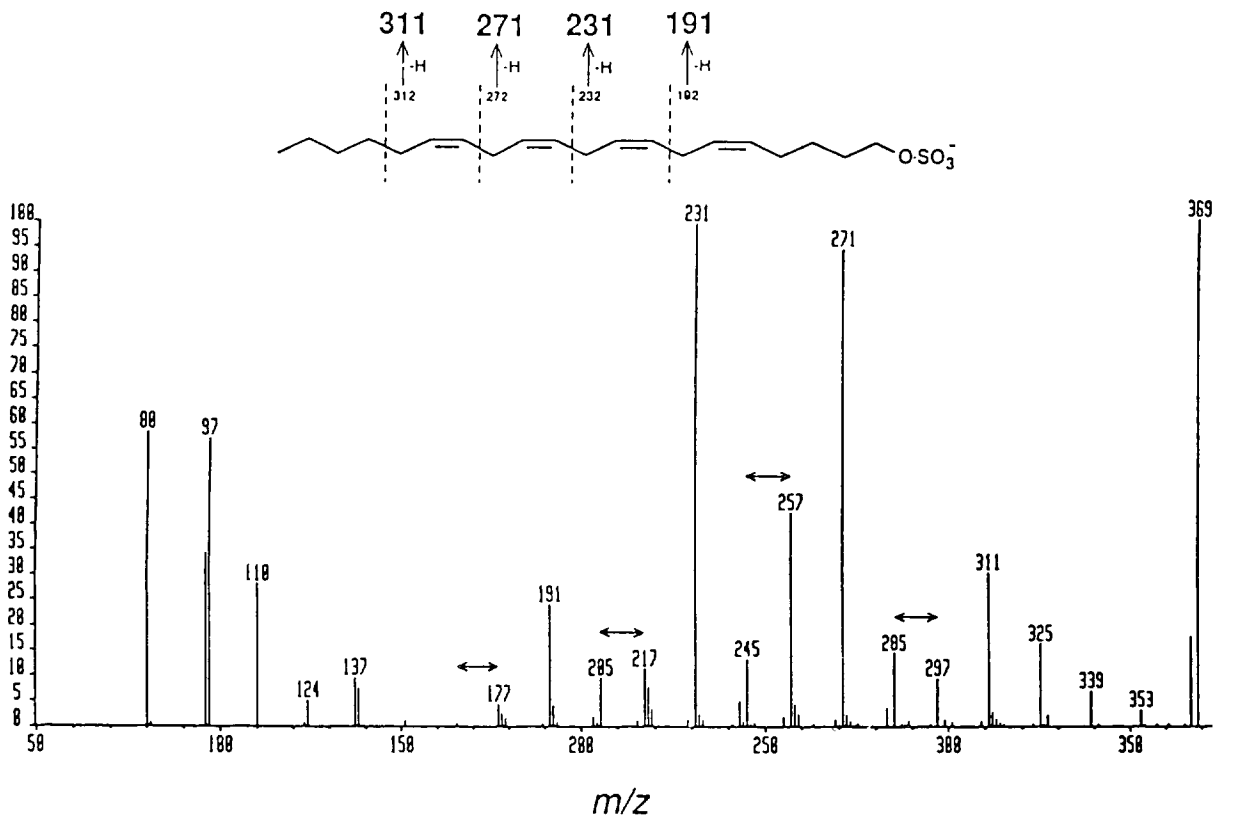

Fig. 2. High-energy negative CID of deprotonated arachidonyl alcohol sulfate ion at m/z 369 using helium collision gas. Major product ions indicated in scheme at top of figure. Reproduced from Ref. [34] with permission.

Cope rearrangement process. Although no single mechanism appeared to predominate, a reasonable approach to the interpretation of these CID spectra was proposed and the CID spectra of unknown compounds could be used to establish the hydroxyl substituent position in relationship to certain sites of unsaturation. Not all double-bond locations were indicated, however. The oxy-Cope rearrangement is specific for the 3-hydroxy-1,5-diene structural unit. These fragmentations may also help clarify the fragmentations observed in the low-energy CID spectra of epoxyeicosatrienoic acids [45]. According to Wheelan et al. [44] the spectrum of each epoxy-substituted eicosatrienoic acid can be thought of as consisting of a summation of two monohydroxy eicosatetraenoic acids. Thus, the spectrum of 11,12-epoxyeicosatrienoic acid contains the fragment ions at m/z 167 and m/z 149, which are two ions indicative of 11-HETE. The epoxy-substituted compound also gives abundant fragment ions at m/z 208 and m/z 179, that are indicative of 12-HETE.

Kim and Sawazaki [10]) have reported the structural analysis of oxygenated polyunsaturated fatty acids by thermospray liquid chromatography–tandem mass spectrometry. The pentafluorobenzyl (PFB) derivatives were analyzed by a sensitive and convenient technique involving filament- or discharge-on negative-ion thermospray followed by low-energy CID of the $[M-PFB]^-$ ions (carboxylate) with argon. The negative-ion fragmentation pattern was examined for various oxygenated polyunsaturated fatty acid standards as well as their deuterated and/or hydrogenated forms. Characteristic fragmentation that occurs at the oxygenated C–C bonds, allows unambiguous determination of the sites of

oxygenation and the required sample amount is typically in the range of 10–20 ng. Fig. 3 demonstrates that the negative CID spectra derived from the $[M - PFB]^-$ precursor ions of 12- and 5-HETE show ions resulting from characteristic cleavage near the hydroxy functional group. Using this method the structures of epoxy, hydroxy derivatives of 4,7,10,13,16,19-docosahexaenoic acid (22:6ω3) formed by soybean lipoxygenase were found to be 13-hydroxy-16,17-epoxy-22:5ω3 and 15-hydroxy-16,17-epoxy-22:5ω3. Hydrogenation to saturated hydroxy-fatty acid derivatives altered the fragmentation pattern in negative-ion MS–MS considerably, although fragmentation still occurred near the oxygenated sites [10]. The daughter ion spectrum of $[M - PFB]^-$ contained a characteristic fragment at m/z 144 which is 3 u higher than that of the corresponding fragment of 12-hydroxy-eicosanoic acid (hydrogenated 12-HETE).

4.1.4. Prostanoids

Many hydroxy-substituted unsaturated fatty acids possess potent biological activities and are effective in vivo at trace concentration. Arachidonic acid serves as the precursor for many of these eicosanoid metabolites. Electron capture ionization GC–MS analysis of the PFB ester, TMS ether derivatives have provided the most sensitive method for molecular mass determination [46–48]. Detailed studies of the EI-MS of several derivatives of prostaglandins (PG) have been reported and libraries of data have been assembled [16,46]. Several hundred metabolites of arachidonic acid have been structurally characterized to date, primarily by mass spectrometry as the major analytical tool [6,16]. This method, however, does not provide structural information, and even low-energy CID of the carboxylate anions from these derivatives does not yield structurally significant ions.

The use of GC–MS–MS has been proposed to increase the specificity and sensitivity of quantitative assays of such eicosanoids, because of the improved signal-to-noise ratio afforded by this technique [49–51]. The detection limit is below 1 pg per injection of the PFB-MO-TMS derivatives analyzed in the selected ion monitoring (SIM) mode by negative-ion chemical ionization (NICI) mass spectrometry. The requirement of extensive sample purification and derivatization is one of the major drawbacks of prostanoid determination by GC–MS and the need for a lengthy purification procedure makes analysis of PG in plasma by GC–MS and SIM too time-consuming. Thus Schweer et al. [50] have used a combination of TLC and GC–MS–MS to determine PGE_1, 15-keto-PGE_0 and PGE_0 in plasma in an isotope dilution assay. The samples along with deuterated internal standards were converted to PFB ester-methoxime-TMS ethers and analyzed by GC–MS–MS on a QqQ-type instrument. In NICI mode the parent ions corresponding to $[M - PFB]^-$ ($[P]^-$) were fragmented by CID and the daughter ions, $[P - (CH_3)_3SiOH]^-$ (PGE_0 and 15-keto-PGE_0) and $[P - 2(CH_3)_3SiOH]^-$ (PGE_1), respectively, were used for quantification. Even though the deuterated compounds had the same molecular mass [($^2H_6$)PGE_1 and ($^2H_4$)PGE_0: 532.4 Da] the two compounds were readily distinguished by means of their CID spectra. They found that plasma concentrations in healthy subjects were about 1–3 pg/ml for PGE_1, 8–17 pg/ml for PGE_0 and 115–205 pg/ml for 15-keto-PGE_0.

Similarly Schweer et al. [50] have developed a method for the determination of PGE_2, 6-keto-$PGF_{1\alpha}$, thromboxane (Tx) B_2 and their metabolites PGE-M (11α-hydroxy-9,15-dioxo-2,3,4,5,20-pentanor-19-carboxyprostanoic acid), 2,3-dinor-6-keto-$PGF_{1\alpha}$, 2,3-dinor-TxB_2 and 11-dehydro-TxB_2 in urine using GC–QqQ-MS and stable isotope dilution. At a signal-to-noise ratio of 5:1, the detection limit is 1–5 pg on column or 10–50 pg/ml of urine, respectively. The great advantage of GC–MS–MS is the requirement of less purification which allows the analysis of 12 urine samples/day by one person. GC–MS–MS quantification of the seven prostanoids per sample occurs in three runs with two or three prostanoids per run. Table 1 gives the masses for the parent and daughter ions of 7 endogenous prostanoids and their deuterated analogues and Fig. 4 shows the daughter ion mass chromatograms for endogenous and deuterium labelled 11-dehydro TxB_2 and PGE-M. It can be seen

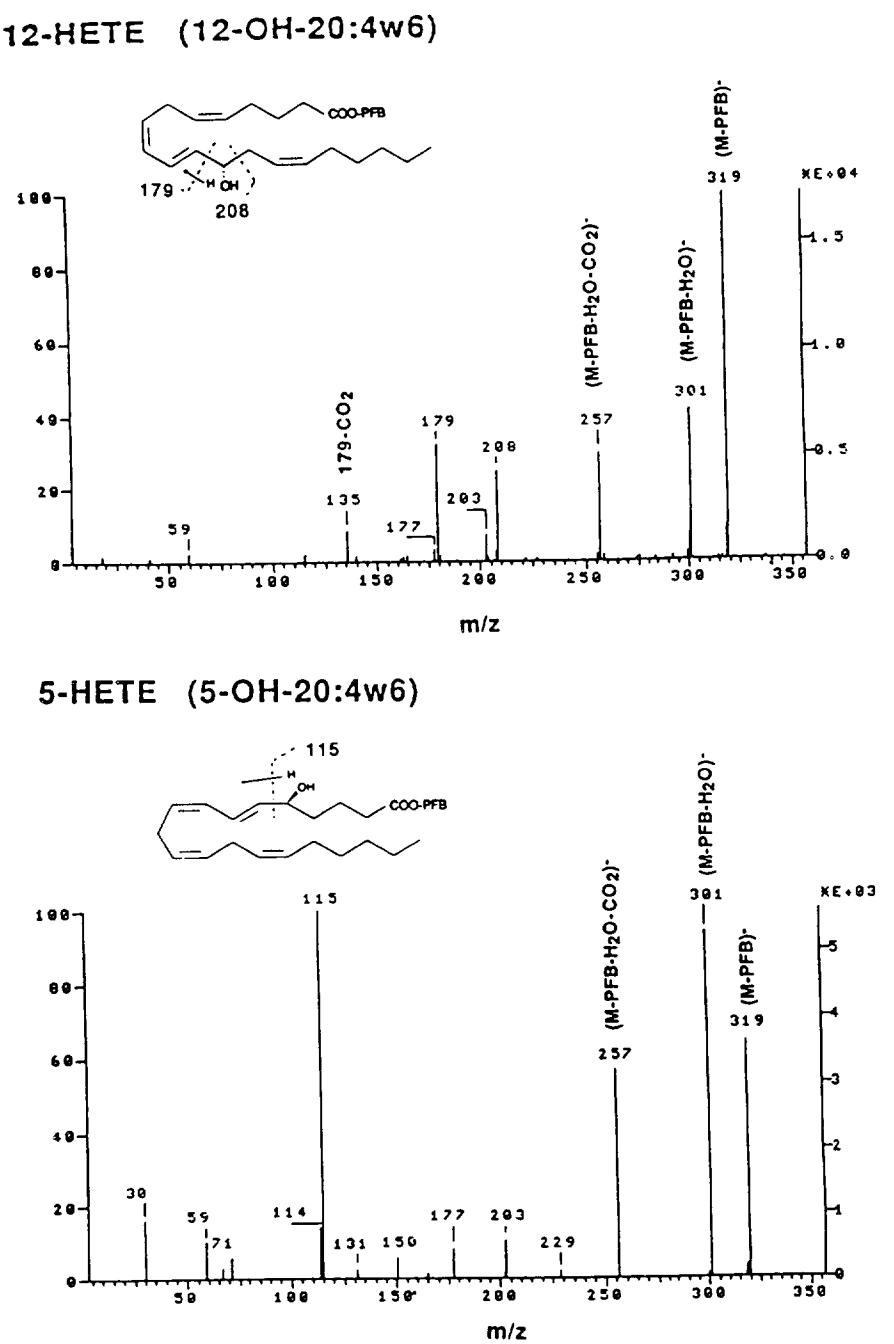

Fig. 3. Low-energy negative daughter ion spectra of [M – PFB] parent ions from the PFB derivatives of 12- and 5-HETE using argon collision gas. Reproduced from Ref. [10] with permission.

Table 1
Parent and daughter ions of endogenous prostanoids and their [^2H_n] analogues ($n = 4$–7) used for quantitation

Prostanoid	m/z (endogenous compound)		m/z (^2H_n-prostanoid)	
	Parent ion	Daughter ion	Parent ion	Daughter ion
2,3-Dinor-6-keto-$PGF_{1\alpha}$	586	240	590	244
6-Keto-$PGF_{1\alpha}$	614	312	618	316
2,3-Dinor-TxB_2	586	240	590	244
PGE_2	524	344	528	348
TxB_2	614	268	618	272
11-Dehydro-TxB_2	511	243	515	247
PGE-M	637	349	644	356

Reproduced from Ref. [50] with permission.

that there is almost no background from the biological samples. PGE-M with its two keto groups forms four methoxime isomers resulting in two broad peaks.

Ferretti and Flanagan [51] have developed an isotope dilution GC–NICI-MS–MS procedure for the analysis of 2,3-dinor-6-oxo $PGF_{1\alpha}$ (PGI_2-M) in urine. The method makes use of solid-phase extraction, pH-controlled partitioning, and TLC. The PFB ester-methoxime-TMS ethers were analyzed by GC–NICI-MS–MS on a QqQ-type instrument by monitoring the natural and deuterium labelled $[P - (3 \times TMSOH + CH_3OH + CO_2)]^-$ daughter ions resulting from the parent $[M - PFB]^-$ ions. Up to 20 samples per day could be done.

Due to a lack of a carboxyl group, some synthetic PG analogues cannot form the PFB ester and another derivative allowing high sensitivity had to be found. Schweer and Fischer [52] report NICI-MS and CID mass spectra of the most abundant ion in the high-mass region of O-2,3,4,5,6-PFB-oxime/TMS/methyl ester derivatives of 6-oxo-$PGF_{1\alpha}$, PGE_1, PGE_2, PGE_3 and 11,16-dihydroxy-9-oxo-16-methyl-prost-13-en-1-oic acid (1-carboxy-rioprostil) and of the PFBO/TMS derivative of 1,11,16-trihydroxy-16-methyl-prost-13-en-9-one (Rioprostil). Except for the 6-oxo-$PGF_{1\alpha}$ derivative, all the NICI (methane) mass spectra show an intense $[M - HF]^-$ parent ion. The CID spectra (Ar, 15 eV) of this parent ion (P^-) showed differences for the *syn* and *anti* oxime isomers. The *syn* isomers have intense $[P - (C_1 - C_7)]^-$ and $[C_6F_4C_2O]^-$ daughter ions, while the most abundant peaks in the spectra of the *anti* isomers are $[P - OH - TMSOH]^-$, $[P - OH - 2TMSOH]^-$ and $[(C_9 - C_{10}) - HF]^-$. The NICI mass spectra of 6-oxo-$PGF_{1\alpha}$ derivatives have intense $[M - HF - (CH_3)Si = CH_2]^-$ ions and the most prominent daughter ions are $[P - (C_1 - C_5)]^-$ and $[P - 2HF - H - (C_1 - C_5)]^-$.

Leukotriene B_4 (LTB_4) is derived from arachidonic acid via the 5-lipoxygenase pathway and is a potent chemotactic mediator for the human polymorphonuclear leukocyte and therefore plays an important role in the inflammatory response [53]. MS–MS of LTB_4 using the *tert.*-butyldimethylsilyl (TBDMS) ether derivative was described by Dawson et al. [54], but little data are available concerning the low-energy collision-induced dissociation of LTB_4 and its related compounds. Fruteau de Laclos et al. [55] have studied the low-energy CID spectra of the carboxylate ions of a series of molecules involved in the lipoxygenase pathway and their stable isotope variants as the PFB ester TMS ether derivatives using a QqQ-type instrument. These molecules included LTB_4, 20-carboxy-LTB_4, 5-HETE and 5-HEA. All CID of the carboxylate anions of these eicosanoids are characterized by losses of small neutral molecules from the derivatizing groups (TMS, TBDMS and PFB) and little fragmentation of the carbon backbone.

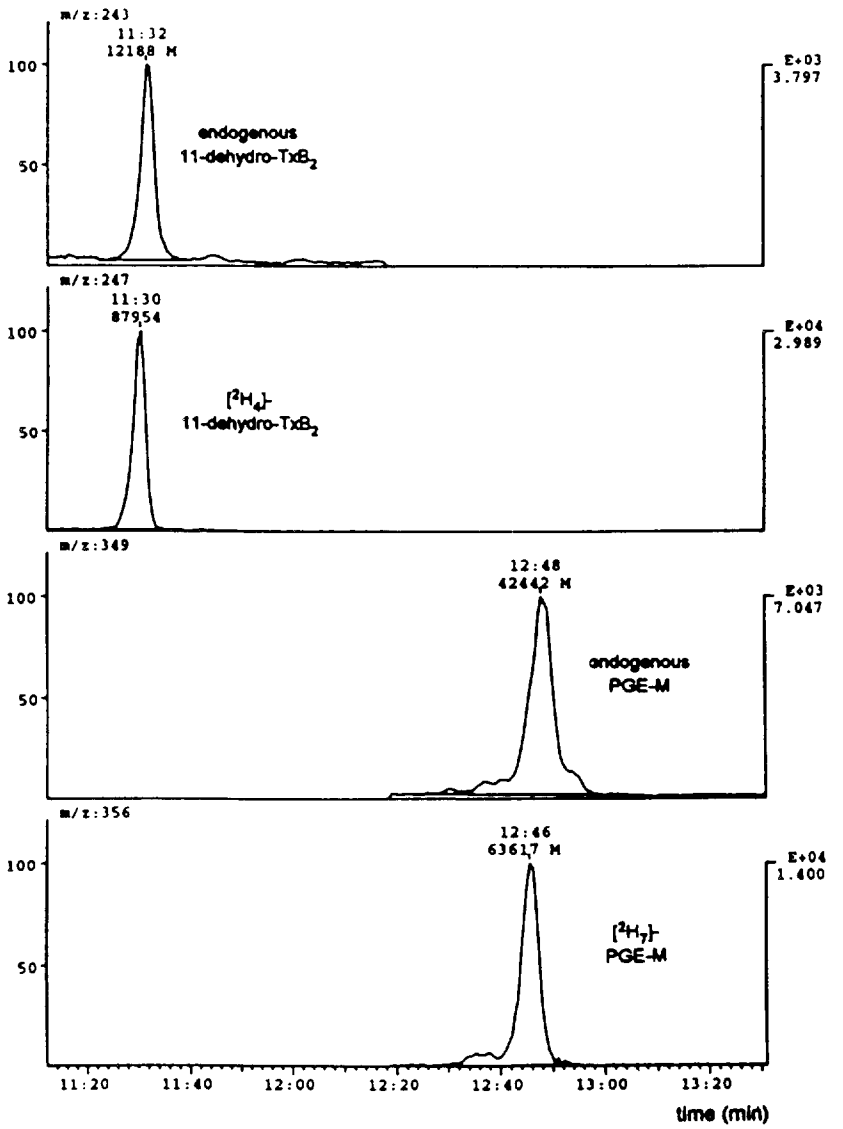

Fig. 4. GC–MS–MS-SIM daughter ion chromatograms of 11-dehydro-TxB2 (0.58 ng/ml) and PGE-M (7.9 ng/ml). Reproduced from Ref. [50] with permission.

Fragmentation resulting from CID of the carboxylate anion of 5-HEA (m/z 399) led to the loss of trimethylsilanol as well as the loss of 146 u corresponding to the loss of trimethylsilanol followed by acrolein, a process specific for 5-hydroxy-containing saturated fatty acids. The major process observed for the 5-HETE carboxylate anion (m/z 391) is the loss of trimethylsilanol by a charge-remote mechanism. For the carboxylate anion of LTB_4 (m/z 479) loss of trimethylsilanol, seen at m/z 389 after CID, results from a complex charge-driven mechanism and not from the expected charge-remote fragmentation mechanism. Quantitation of LTE_4 via CID of the carboxylate ion of 5-HEA (PFB, TMS) has also been reported [56].

Negative-ion formation by FAB of underivatized LT, PG and other eicosanoids results in

abundant carboxylate anions, and CID-MS–MS of these ions produces structurally significant products [40,41,42]. Alternately FAB analysis of underivatized PG has been carried out as positive ions using lithium or barium salts [40]. For example CID of the carboxylate anion from $PGF_{2\alpha}$ (m/z 353) yields ions at m/z 335, 317 and 299 resulting from the loss of one to three molecules of water and also a very abundant ion at m/z (353-44) resulting from the loss of C_2H_4O rather than the expected CO_2 [57]. This loss of C_2H_4O was found to be characteristic of eicosanoids possessing the 1,3-diol structure such as that found in thromboxane B_2 as well as $PGF_{2\alpha}$ [58] and has been used to aid in the characterization of isoprostane phospholipids isolated from rat liver following exposure of the rat to carbon tetrachloride in vivo [59]. Other PGs, which have different functionalities in the cyclopentane ring, do not readily lose C_2H_4O.

The LTC_4, LTD_4, and LTE_4 are sulfur-containing eicosanoids that are conjugated respectively to glutathione, cysteinylglycine or cysteine, through a thioether bond. They are potent constrictors of various smooth muscles and were initially identified as the mediator slow-reacting substance of anaphylaxis [60]. These non-volatile eicosanoids and many of their derivatives are readily analyzed by FAB-MS [16]. Positive and negative FAB of these compounds yield abundant $[M+H]^+$ and $[M-H]^-$ ions, respectively. Sala et al. [42] and Raftery et al. [41], who have studied the CID of the cysteine leukotrienes, found that either high- or low-energy CID of LTC_4 and LTD_4 yields fragment ions largely containing only the peptide portion of the molecule. However, it can be seen in Fig. 5 that CID of either the positive or negative high-mass parent ion of LTE_4 yields abundant fragment ions, which retain the arachidonate portion of LTE_4. Thus CID of the $[M-H]^-$ anion of LTE_4 at m/z 438, yields an abundant thiol carboxylate anion at m/z 351, resulting from loss of the cysteine portion of the ion. As a demonstration, the presence of intact LTE_4 in human urine was established by showing that the CID spectrum for the parent ion at m/z 438 from LTE_4 partially purified by reversed-phase HPLC from human urine, was identical with that of a synthetic standard [42].

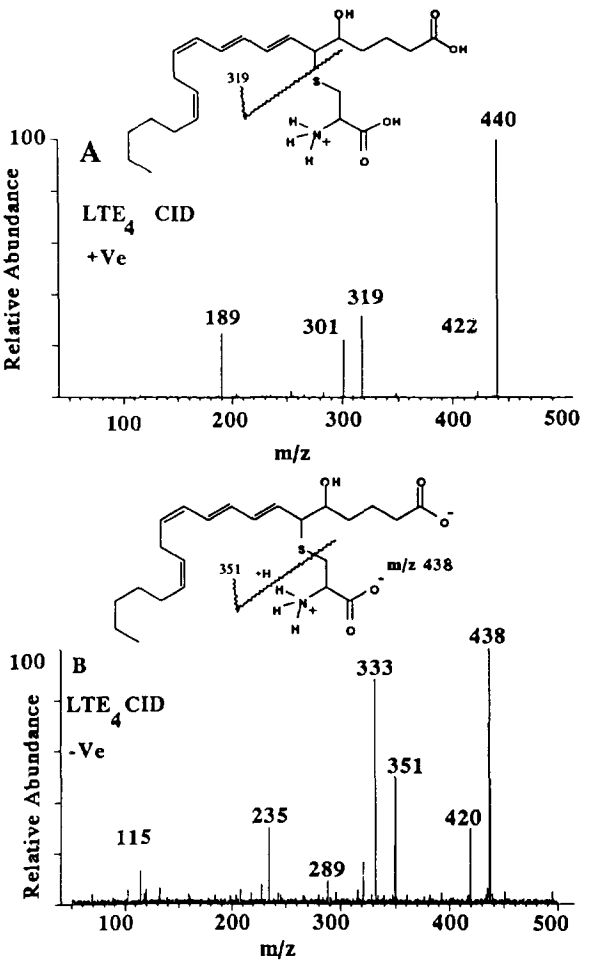

Fig. 5. Low-energy CID spectra of the positive and negative molecular ion species obtained by FAB of LTE_4. (A) CID of $[M+H]^+$ at m/z 440. (B) CID of $[M-H]^-$ at m/z 438 [16]. The spectra were obtained on a QqQ-type instrument at 30 eV collision energy using argon as collision gas (67 Pa). Reproduced from Ref. [16] with permission.

Mamer et al. [61] have found that the response in FAB or continuous-flow liquid secondary ion MS (CF-LSIMS) is significantly enhanced by converting the free amino group of LTE_4, LTD_4, and LTC_4 to a 5-triphenylphosphoniumvaleryl-amide (TPPV). The molecular cations were prominent features of the positive FAB and CF-LSIMS spectra of the TPPV derivatives and it was possible to detect 3 pg of LTD_4 with capil-

lary HPLC inlet CF-LSIMS and SIM monitoring. The TPPV derivatives are also more stable to chemical degradation than the native PLTs.

Other more complex lipids, such as oxidized cellular phospholipids (see below) are known to be formed in whole cells. These and other oxidized lipids may play a role in the development of atherosclerosis by modifying LDL so that it is taken up by macrophages instead of the normal LDL receptor [62]. Isoprostanes are a class of arachidonic acid autooxidation products that have recently been detected in plasma, urine and tissue samples [48]. There are approximately 16 possible isomers and their structures have not yet been determined in the complex mixtures produced in vivo [6]. Also very little is known about the involvement of other polyunsaturated fatty acids as precursors of additional lipid mediators. There is evidence that such mediators exist and may play an important role in cell signalling events (e.g. PG-like substances derived from fish oil fatty acids).

Because subpicomolar detection limits can now be achieved by MS–MS procedures, it is competitive with other assays including radioimmunoassay and enzyme immunoassay. Mass spectrometric quantitation is becoming accepted as a necessary step for unequivocal validation of the occurrence of eicosanoids in biological systems.

4.2. Complex esters

4.2.1. Neutral glycerolipids

On-line LC–MS with chemical ionization has been an effective general method of identifying the components of a triacylglycerol (TG) mixture since both retention times and molecular mass information can be obtained [64,65]. More recently the analysis of TG has been made by FAB mass spectrometry and CID-MS–MS. Evans et al. [66] reported the early results on optimizing the information yielded by FAB-MS–MS. The samples analyzed were the commercially available 1,2-dioleoyl-rac-glycerol, 1,2-dioleoyl-3-palmitoyl-rac-glycerol, 1,3-dioleoyl-2-palmitoyl-rac-glycerol, 1-palmitoyl-2-oleoyl-3-stearoyl-rac-glycerol and others. Structural characterization of the monoacylglycerols (MG), diacylglycerols (DG) and TG was achieved by means of positive- and negative-ion FAB-MS combined with CID. Only $[M-(R-COO)]^+$ and $[R-COO]^-$ ions without any significantly abundant pseudo-molecular ions were produced by FAB of the neat samples, without any liquid matrix, but samples dissolved in m-nitrobenzoyl alcohol solution containing NaI gave rise to abundant $[M+Na]^+$ species together with the $[M-(R-COO)]^+$ ions which afford the identification of the constituent acyl groups. CID of the $[M+Na]^+$ cations was found to be useful for the analysis of complex glyceride mixtures even though it led to a similar production of $[M-(R-COO)]^+$ and $[M-(R-COO)+Na]^+$ ions. The authors also made a preliminary attempt at analyses of olive oil and other seed oils but due to inadequate mass resolution, CID-MIKES alone did not yield information about the level of unsaturation in the fatty acid fragments and the oils had to be further analyzed using B/E linked scans on the parent ions. Constant B/E scans provide spectra with higher mass resolution but also exhibit artifact peaks which confound interpretation. Therefore the preliminary analysis of olive and seed oils was made with both CID-MIKES and constant BE scans to complement each other.

Kallio and Currie [3] have introduced a tandem mass spectrometric method for the analysis of fats and oils based on NH_4/NICI of the TG and subsequent analysis of the TG ion mixture by MS–MS using a QqQ instrument. This method allows for the determination of the molecular mass species, the fatty acid composition of these species, and some information about the fatty acid located at the *sn*-2-position. The first step of the tandem mass spectral analysis is the CI of the TG with ammonia as the reagent gas which produces a complex mixture of ions within the ion source of the mass spectrometer. The molecular species are thus resolved by molecular mass by virtue of the $[M-H]^-$ ions, but a single mass seldom represents one unique species of TG. Therefore each of these ions is subjected to CID, that produces $RCOO^-$, $[M-H-RCOOH-100]^-$, $[M-H-RCOOH-74]^-$ and $[M-H-$

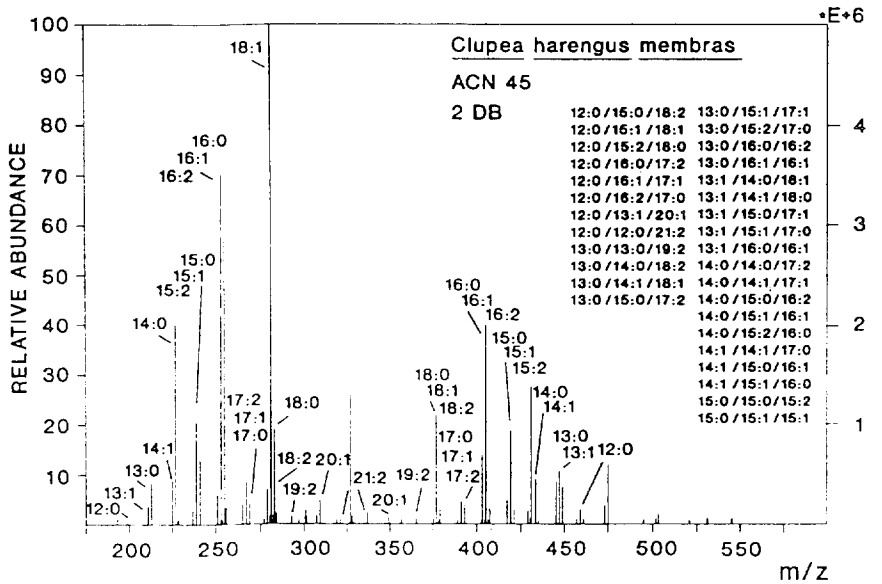

Fig. 6. Low-energy CID spectra of the [M − H]⁻ parent ion at *m/z* 759.7 from the triacylglycerols in Baltic herring (*Clupea harengus membras*) fish oil. NICI mass spectra were obtained on a QqQ-type instrument using ammonia as reagent gas and CID were obtained at 15 eV collision energy using argon as collision gas (240 Pa). Reproduced from Ref. [3] with permission.

RCOOH]⁻ daughter ions that are used to identify the individual molecular species. Fig. 6 shows the complex CID daughter ion spectrum of the *m/z* 759.7 parent ion for the TG with 45 acyl carbon number and two double bonds from Baltic herring (*Clupea harengus membras*) fish oil. This molecular mass fraction consists of 20 fatty acids and 31 TG species [3]. The fatty acids composition of each TG mass can be rapidly established by means of the corrected intensity of the $RCOO^-$ ions and the combinations of fatty acids that comprise the TG species can then be calculated by fitting the fatty acid composition to the molecular mass of the TG. The details of these calculations can be found in the review by Kallio and Currie [3].

The NICI-MS–MS method also allows distinction between the *sn*-1/3 and *sn*-2-positions of the TG. In the CID of the TG ions, the formation of $[M - H - RCOOH - 100]^-$ fragment ions that originate from the *sn*-2-position is strongly reduced but not entirely eliminated. It is possible that the small amount resulting from the *sn*-2-position is a result of acyl migration [67], which is presumably caused by the method of sample introduction into the mass spectrometer (volatilization from a heated probe). It is then possible to determine the proportion of AAB- and ABA-type TG species in a simple binary TG mixture. The determination of the fatty acids located in the *sn*-2-position of TG in fats and oils is generally more difficult. Natural fats and oils may consist of up to 30 fatty acid combinations of TG but Kallio and Currie [3] have described certain aids that can help in the calculation. The MS–MS method is a powerful and fast tool, when proportions of fatty acid combinations from molecular mass fractions of different fats and oils are to be compared. Kallio and Currie [3] have compiled results from a series of experiments.

Kallio and Currie [67] have used NICI-MS–MS to analyze the TG of low erucic acid rapeseed oil. In total, 44 FA combinations of TG were identified in the oil, of which more than half existed in trace amounts (<1%). One new finding was that the major TG source of stearic acid in the oil was 18:1–18:0–18:1 and that the stearic acid is preferentially located in the *sn*-2-position. The MS–MS spectra showed that most molecular mass fractions consisted of mixtures of

TG, thus restricting the investigators' ability to evaluate the regiospecific positions of fatty acids in the TG. Nevertheless, it was possible to determine that in many TG certain fatty acids were preferentially (>67%) located at the *sn*-2-position.

Currie and Kallio [68] have applied the rapid MS–MS method using ammonia NICI [67] to identify the most abundant fatty acid combinations occurring in the TG of human milk. They also obtained information concerning the fatty acyl groups located preferentially at the *sn*-2-position. Altogether 29 $[M-H]^-$ ions originating from TG composed of eleven major fatty acids were further analyzed by CID for daughter ions. The 29 MW species analyzed by MS–MS indicated that these MW fractions comprised 277 different TG, even when the positional isomers and different fatty acid isomers were excluded. The major fatty acid combinations in TG were determined, as 12:0–12:0–16:0, 12:0–16:0–18:1, 16:0–18:1–18:1, 16:0–18:2–18:1, 18:2–18:1–18:1 and 18:2–18:2–18:1, and the most abundant TG was 18:1–16:0–18:1, which made up about 10% of the total. For example, Fig. 7 shows the CID spectrum of the $[M-H]^-$ precursor ion at *m/z* 789 for the species having 47 acyl carbons and one double bond. Two main groups of ions are shown: the low-mass group of $[RCO_2]^-$ ions and the mid mass group of $[M-H-RCO_2H-100]^-$ ions. Using this data the authors have identified 22 molecular species made up of 15 different fatty acids. The MS–MS results are consistent with the currently accepted concept that in human milk TG 16:0 is typically located at position *sn*-2. It could be seen that in several instances, where 16:0 acid was absent, 14:0, 15:0 and 12:0 preferentially took its place. In order to accurately calculate the proportions of fatty acids and their positional distribution in the TG species, precise calibration for all the $RCOO^-$ ions is required.

The analysis of TG in human milk has been continued by Kallio and Rua [69]. In this NICI-MS–MS study the SIMPLEX method and a simple linear model were used to interpret the distribution of fatty acids between *sn*-2- and *sn*-1(3)-positions in 24 major molecular mass groups of TG. The SIMPLEX method guarantees that the solution is optimum but not necessarily

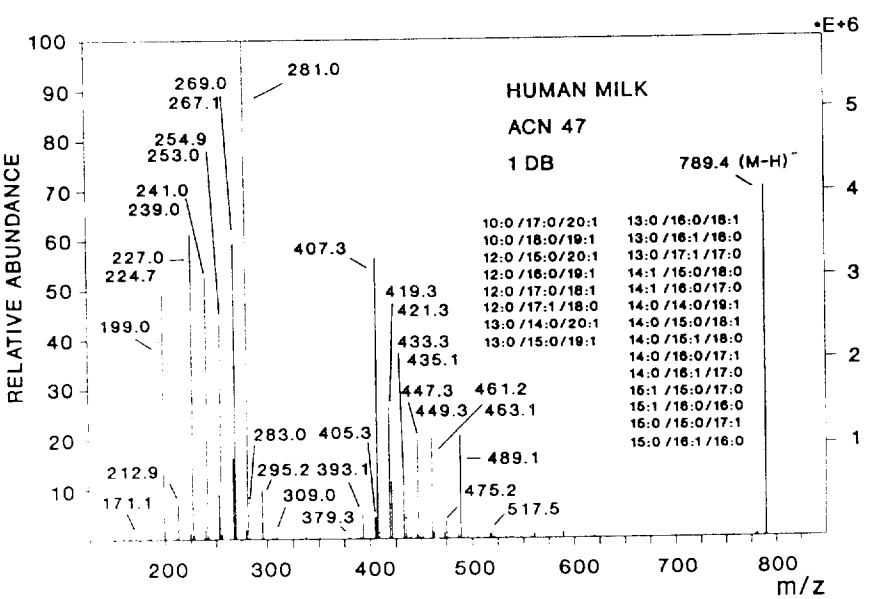

Fig. 7. Low-energy CID spectra of the $[M-H]^-$ parent ion at *m/z* 789.4 from the triacylglycerols in human milk. NICI mass spectra were obtained on a QqQ-type instrument using ammonia as reagent gas and CID were obtained at 15 eV collision energy using argon as collision gas (240 Pa). Reproduced from Ref. [3] with permission.

unique. The positions of the three fatty acids in a TG molecule were shown to depend on the fatty acid composition. The MS–MS data essentially confirmed the results of earlier analyses of the molecular species of human milk fat on the basis of chromatographic and stereospecific analyses, although there were some exceptions. Since human milk is synthesized by a multistage process utilizing a variety of endogenous fatty acid sources, the various molecular associations and positional distributions of the fatty acids do not necessarily represent any one specific biosynthetic process or fatty acid and positional specificity.

In order to simplify the MS analysis of very complex mixtures it is necessary to use some chromatographic procedure to fractionate the sample prior to MS–MS analysis. Accordingly Laakso and Kallio [70,71] used silver ion HPLC to separate winter butterfat into six fractions differing in their degree of unsaturation. In addition they analyzed each of the fractions by reversed-phase HPLC and found that the monoenoic TG containing oleic acid (18:1, *cis* ω-9) and 18:1 *trans* (mainly vaccenic acid (18:1ω-7, *trans*)) were isolated in two fractions. Thus, they were able to identify certain species according to the presence of *cis* or *trans* 18:1. In the future the characterization of the fatty acids in the *sn*-2-position may be facilitated by interfacing chromatographic procedures to the MS–MS system, e.g. argentation HPLC or supercritical fluid chromatography (SFC).

One aspect of tandem mass spectrometry that needs to be developed further is MS–MS–MS. Such a technique would allow the fatty acid fragment ions, $RCOO^-$ (produced from the CID of $[M-H]^-$) to be fragmented further. It may be possible that the CID spectra of these $RCOO^-$ ions can be used to determine the structure and position of the double bonds. Although low-energy CID spectra, as in the examples given in this section, have so far failed to produce this information from fatty acids, it has been shown that this data is readily available from high-energy CID spectra, e.g. from sector mass spectrometers.

Duffin et al. [72] have introduced an MS–MS method based on the analysis of the $[M+NH_4]^+$ ions produced in an ionspray (pneumatically assisted ES) source. The paper presents ES and tandem mass spectrometric analysis of synthetic mixtures containing MG, DG, and TG. The analyses were made with a QqQ mass spectrometer having an atmospheric pressure ion source and an ionspray interface. Samples were dissolved in chloroform–methanol (70:30), which was modified by the addition of alkali-metal or ammonium salts, or by addition of formic acid to favour the addition of a cationic species to the sample molecule. However, the ammonium adducts were used for the MS–MS studies because the sodium adducts were resistant to fragmentation by CID. ES analysis of acylglycerol standards yielded $[M+NH_4]^+$ ions, with no fragmentation, for any species that were present at low pmol/μl concentrations. Acylglycerols that contained unsaturated fatty acid chains were observed to exhibit a response in the mass spectrum greater than those with saturated chains, and the response resulting from the molecular adduct ions of the acylglycerols decreased in the order MG > DG > TG. CID of the $[M+NH_4]^+$ ions yielded good quality spectra that contained an abundance of information. At 50 eV collision energy ions corresponding to $[M-RCOO]^+$ were the main CID fragmentation products along with some acylium ions. If the collision energy was 130 eV, fragment ions resulting from dissociation of the carbon–carbon bonds of the fatty acids appeared in the low-mass region of the spectra, but locations of double bonds could not be distinguished because the site of unsaturation apparently migrated during the collisions. Fig. 8 shows the MS–MS product ion spectra from ammoniated mono-, di- and tripalmitoylglycerol [72]. CID of the $[M+NH_4]^+$ ion of monopalmitoylglycerol yielded $[M+H]^+$, $[MH-H_2O]^+$ and RCO^+ ions as well as some low-mass acylium or hydrocarbon ions resulting from cleavage of fatty acid carbon–carbon bonds. Similarly CID of the $[M+NH_4]^+$ ion from either dipalmitoyl or tripalmitoylglycerol yielded $[MH-H_2O]^+$ and RCO^+ ions as well as some low-mass acylium or hydrocarbon ions resulting from cleavage of carbon–carbon bonds in fatty acid chains.

Since acylglycerols that contain unsaturated

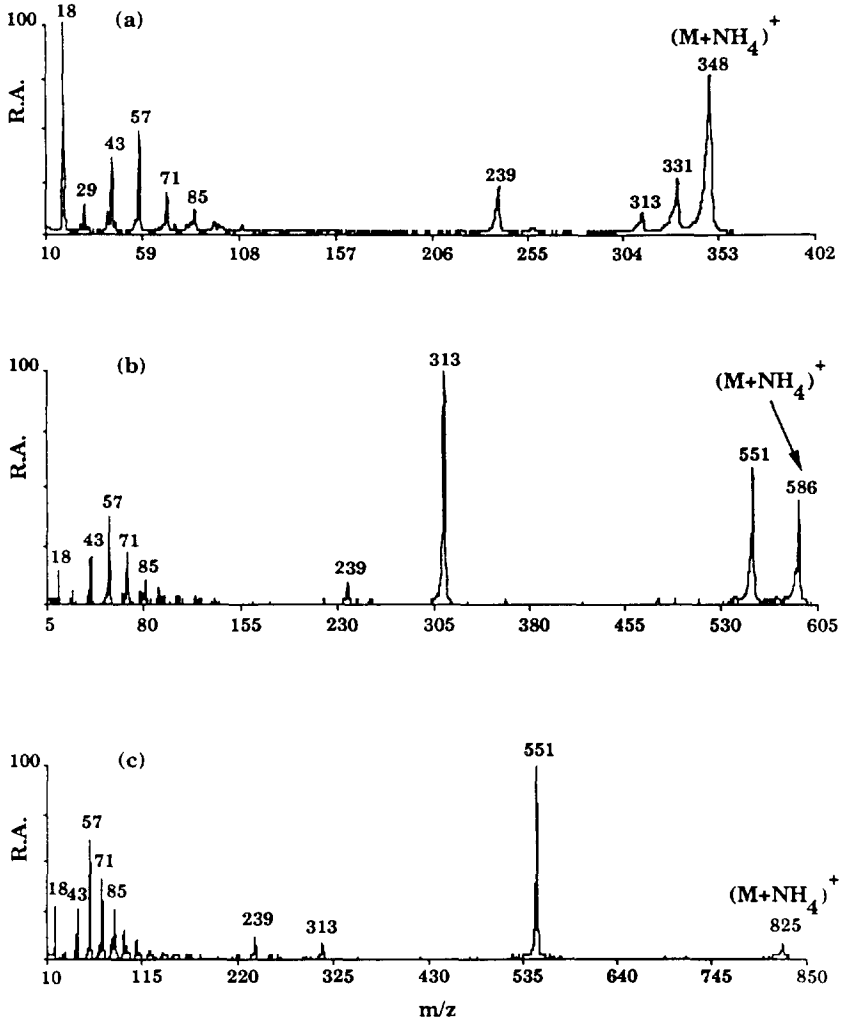

Fig. 8. Comparison of the MS–MS product ion spectra of ammoniated monopalmitoyl (a), dipalmitoyl (b) and tripalmitoyl (c) glycerols acquired at 130 eV collision energy. Reproduced from Ref. [72] with permission.

fatty acids are cleaved at the same bond locations as acylglycerols containing saturated fatty acids, the degree of unsaturation of individual fatty acids that comprise an acylglycerol can be determined by the mass of the product ions in the MS–MS spectrum. Positional data could not be obtained because there was no preferential cleavage of acyl chains from the *sn*-1(3) or *sn*-2-position of the TG.

The ES analysis of an unknown lipid extract isolated from a mammalian cell culture reactor yielded $[M + NH_4]^+$ ions for a diverse mixture of TG species and MS–MS analysis of these ions revealed the presence of appreciable amounts of 14:0, 16:0, 16:1, 18:0 and 18:1 component fatty acids. In general the MS–MS data agreed with the fatty acid composition generated by GC–FID but the relative amounts of the TG species could not be accurately deduced from the ES data because of the dependence of ion current abundance on analyte polarity, including unsaturation.

It is possible to conduct pseudo MS–MS experiments using HPLC coupled to a pneumatical-

ly assisted ES (ionspray) and a single Q mass spectrometer (J.J. Myher, A. Kuksis and L. Marai, 1994, unpublished results). Ions formed in the electrospray source can be subjected to CID by increasing the voltage (V_{CapEx}) between the capillary exit and the first skimmer. Fig. 9 shows plots of the pseudomolecular $[M + NH_4]^+$ ion at m/z 874.8 and two $[M - RCOO]^+$ fragment ions at m/z 577.6 and 601.6 in the ES spectrum of 1-palmitoyl-2-oleoyl-3-linoleoyl-*sn*-glycerol plotted against V_{CapEx}. For V_{CapEx} between 170 and 210 V there is very little fragmentation compared to the $[M + NH_4]^+$ ion intensity. For a V_{CapEx} of 250 V the $[M + NH_4]^+$ ion has almost disappeared and the $[M - RCOO]^+$ fragment ions are at their maxima. Fig. 10A shows the ES mass spectrum of 1-palmitoyl-2-oleoyl-3-linoleoyl-*sn*-glycerol taken with $V_{CapEx} = 200$ V. At this voltage there is a low abundance of the $[M - RCOO]^+$ ions at m/z 575, 577 and 601. Along with the base peak due to the $[M + NH_4]^+$ ion at m/z 874 there is also a small amount of $[M + Na]^+$ ion at m/z 879. At $V_{CapEx} = 170$ V there are only $[M + NH_4]^+$ and $[M + Na]^+$ ions present. Fig. 10B shows the ES mass spectrum of 1-palmitoyl-2-oleoyl-3-linoleoyl-*sn*-glycerol taken with $V_{CapEx} = 250$ V. The $[M - RCOO]^+$ ions now dominate the spectrum and $[M + NH_4]^+$ ions have almost disappeared. The $[M + Na]^+$ ion appears to be more stable and now dominates the high-mass end of the spectrum.

4.2.2. Glycerophospholipids (GPL)

GPL are found in abundance in the cell membranes of living organisms. Much of the MS–MS research has recently centred around the platelet activating factor (PAF), the use of GPL as biomarkers for bacteria and algae, and the determination of general membrane function in living matter. The resurgence of interest in the mass spectrometry of GPL is due primarily to the development of FAB ionization and MS–MS. FAB allows the non-volatile GPL to be analyzed without prior derivatization, and MS–MS allows GPL mixtures to be fully characterized without

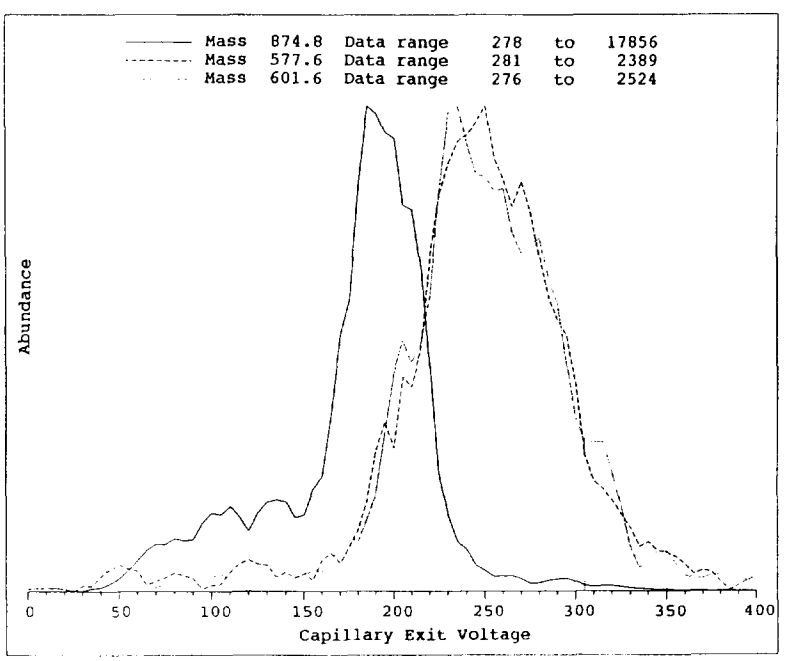

Fig. 9. Plots of ion abundance versus capillary exit voltage for $[M + NH_4]^+$ (m/z 874.8) and two $[M - RCOO]^+$ (m/z 577.6 and 601.6) ions from 1-palmitoyl-2-oleoyl-3-linoloeyl-*sn*-glycerol. Instrument: HP 5985 quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray source. Sample dissolved in methanol–isopropanol–aq. ammonia (40:60:0.2) and introduced into the ion source at a flow-rate of 50 μl/min. Reproduced from J.J. Myher, A. Kuksis and L. Marai, 1994, unpublished results.

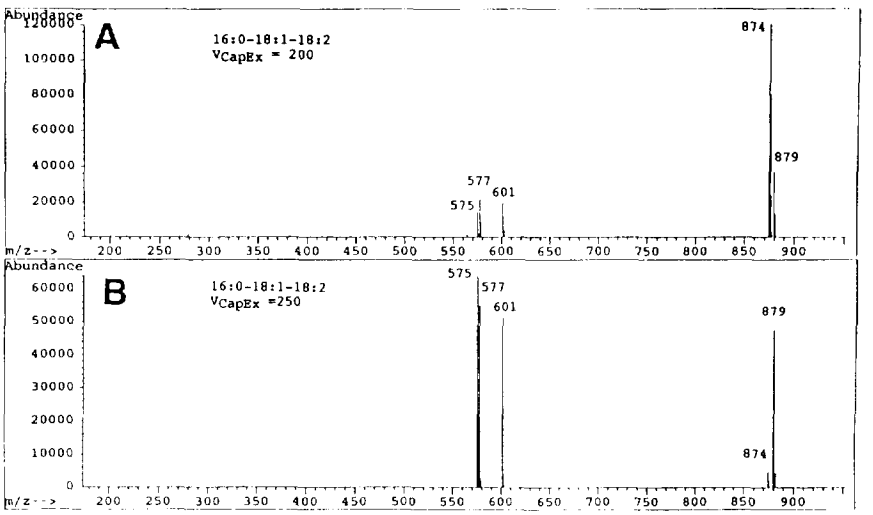

Fig. 10. ES mass spectra of 1-palmitoyl-2-oleoyl-3-linoloeyl-*sn*-glycerol at two capillary exit voltages: (A) $V_{CapEx} = 200$; (B) $V_{CapEx} = 250$. Instrument: HP 5985 quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray source. The sample was eluted by C_{18} reversed-phase HPLC using a flow-rate of 0.85 ml/min and a gradient of 20 to 80% isopropanol in methanol. A flow of 0.15 ml/min isopropanol containing 1% ammonia was added post-column. Reproduced from J.J. Myher, A. Kuksis and L. Marai, 1994, unpublished results.

prior separation. While most of the GPL information is contained in the conventional FAB-MS spectrum, the data are difficult to interpret: signals from lipid species at low concentrations are often buried in the high chemical background associated with FAB-MS, and FAB-MS does not work well with mixtures of GPL. FAB-MS–MS analysis of daughter ions produced by CID of molecular ion precursors has proven useful for GPL structure analysis [73–75]. FAB in conjunction with CID provides spectral data pertaining to the polar head group, the acyl substituents and to some extent the positional placement of the fatty acyl chains [76]. While this method is useful for individual GPL, screening of mixtures would require mathematical analysis of data composed of the daughter spectrum from the ion current at every mass number. Constant neutral-loss scanning for polar head functional groups has been shown to be very useful for detection and differentiation of GPL classes in complex matrices and mixtures [77].

When GPL ions undergo low-energy CID, the few different ions formed are the result of cleavages at specific points, which are common to all classes of GPL. By taking advantage of this fact, Cole and Enke [78], who were interested in identifying microorganisms, have developed a general scheme that uses a QqQ mass spectrometer to rapidly characterize the GPL content and structures present in crude lipid extracts. The scheme is based first on FAB ionization of a crude lipid extract. Then the combination of unimolecular positive-ion constant neutral-loss and parent scans provide independent diagnostic mass spectra for each of many specific GPL classes, while negative-ion daughter scans provide the empirical formulae and positions of the fatty acyl constituents on each GPL. An extensive GPL screening on a single sample is possible when the tandem mass spectrometer is programmed accordingly. The procedure is sensitive and the spectra are reproducible, and the response is linear over at least a five-logarithm range of cell concentrations. With the exception of PC the major reaction occurring in the positive mode is cleavage of the glycerolphosphate bond, resulting in the production of a DG-type ion. Phosphatidylcholine (PC) fragments so that the head group usually retains charge or is lost as a sodium adduct, which causes difficulties in spectra interpretation. A fragment cation of *m/z*

184 is used for the characterization of PC. In the negative-ion mode, the carboxylate ions are the major CID products. The fact that GPL always cleave around their functional groups led Cole and Enke [78] to develop a general scheme for the rapid characterization of GPL classes present in microorganisms which includes information on the fatty acyl chains present on the molecular species of each class.

The negative-ion daughter spectra can be used to assign the positions of the two fatty acyls on the GPL. Jensen et al. [73] have postulated preferential formation of the carboxylate anion from the fatty acid at the *sn*-2-position (closest to the head group) over that of the fatty acid at the *sn*-1-position. Murphy [16] has reviewed the data from different laboratories. The negative FAB spectra of PC have high-mass ions corresponding to $[M-15]^-$, $[M-60]^-$, $[M-87]^-$, as well as an adduct ion involving the FAB matrix compound. The CID spectrum of the $[M-15]^-$ and $[M-60]^-$ ions consists primarily of carboxylate ions and it has been shown that for species containing up to three double bonds the abundance is highest for the carboxylate ions originating from the *sn*-2 position of the PC. The observed difference in the CID spectrum of the $[M-87]^-$ ion for the abundance of the fragments resulting from loss of a fatty acid (RCOOH) moiety is more consistent. The abundance is always greater for loss of the fatty acid in the *sn*-2 position [79,80]. These results apply to both low- and high-energy processes [16] and may apply to other GPL [79]. As described previously, high-energy CID of the carboxylate ions can be used to determine the location of double bonds [81] or other substituents on the fatty acid chains of the GPL molecule.

Kayganich and Murphy [80] have used negative FAB-MS–MS to identify the composition of the arachidonic-containing molecular species of diacyl GPC from human polymorphonuclear leukocytes. This was done by scanning for the precursor ions of the arachidonic acid carboxylate ion at m/z 303. From the molecular masses determined from the mass of the precursor ions one can then calculate the mass of the other fatty acid of the diacyl GPC. Alkylacyl GPC species are isobaric with diacyl GPC species having an odd number of carbon atoms. They can be differentiated by the fact that there is only a single carboxylate anion produced from the alkylacyl GPC by CID of the corresponding $[M-15]^-$ ion [82]. Further confirmation is obtained by CID of the $[M-86]^-$ ion which produces a strong neutral loss typical of ether-linked molecular species. The ethanolamine GPL, which consist of diacyl, alkylacyl and alk-1-enylacyl subclasses were also determined by generating parent ion scans for the arachidonic acid carboxylate anion [83]. However, it was not possible to differentiate between alk-1-enylacyl and isobaric unsaturated alkylacyl species by FAB-MS–MS alone. This problem was solved by repeating the FAB-MS–MS procedure after selectively hydrolyzing the alk-1-enyl bond by mild acid hydrolysis. The plasmalogens which were converted to 1 lyso PE and fatty aldehydes do not have $[M-H]^-$ ions within the mass range scanned.

Chen et al. [84] have used negative FAB-MS combined with MIKES analysis to characterize the GPL from rabbit kidney. Each lipid class was first separated into molecular species by reversed-phase HPLC and then the species were characterized by FAB-MS–MS. As shown earlier it was possible to establish the positional placement of the fatty acyl chains on the basis of the intensities of the $[M-\text{choline}-R_2COOH]^-$ and $[M-\text{choline}-R_2CO-H]^-$ ions derived from the $[M-\text{choline}]^-$ ion. It was also shown that this approach could be extended to the analyses of PA, PE, PS, and PI. They identified 11 arachidonyl-containing species of GPL in addition to 17 other molecular species. The PE fraction was shown to be made up of both diacyl- and alk-1-enylacyl-GPE species.

Kerwin et al. [85] have described positive- and negative-ion ES-MS–MS to identify PC, PE, PI, and PS headgroups and their alkyl, alk-1-enyl and acyl constituents. The ES spectrum of GPC showed mainly $[M+H]^+$ in the positive mode and $[M-H]^-$ in the negative mode. CID of the $[M-H]^-$ ion produced carboxylate ions which were used to determine the molecular species composition. The ES spectra of bovine PE (Fig.

11) show dominant ions for $[M + H]^+$ in the positive mode and $[M - H]^-$ in the negative mode. MS–MS of diacyl GPE is illustrated in Fig. 12, which shows the CID spectra for the 16:0–22:6 species of the $[M + H]^+$ ion at *m/z* 792 and the $[M - H]^-$ ion at *m/z* 790. CID of $[M + H]^+$ produced $[M - \text{phosphoethanolamine}]^+$ at *m/z* 651 and $[M - 181]^+$ at *m/z* 610 and an ion at *m/z* 181, which corresponds to phosphorylethanolamine plus the carbon skeleton ($-CH_2CHCH_2-$) of glycerol. CID of the $[M - H]^-$ ion produced the carboxylate ions at *m/z* 283 and 327 corresponding to 18:0 and 22:6, respectively. Alk-1-enylacyl species behaved in like fashion in the negative mode but in the positive mode CID of $[M - H]^-$ produced fragments that the authors claimed were due to loss of the *sn*-2 acyl group, loss of ethanolamine and formation of a phosphonium ion. However, it still was not possible to differentiate between isobaric alkylacyl and alk-1-enylacyl species. ES spectra of PS and PI both showed $[M + H]^+$ and $[M + Na]^+$ ions in the positive mode and $[M - H]^-$ ions in the negative mode, while CID of the $[M - H]^-$ ion produced carboxylate ions. In general, Kerwin et al. [85] found no information indicating the location (*sn*-1 or *sn*-2) of the two acyl chains in the diacyl species. ES-MS–MS does however provide a rapid, sensitive and quantitative alternative to previously published methods for analysis of molecular species. According to Kerwin et al. [85] the presence of detergents and phosphate ions may interfere with the ES analysis.

Han and Gross [86] have used electrospray ionization and a QqQ-type mass spectrometer to determine the individual molecular species in sub-picomole quantities of human erythrocyte GPL and sphingomyelins (SPH). It was demonstrated that SPH and PC were most effectively analyzed as the sodiated positive ions, whereas PE and the negative GPL were best analyzed as

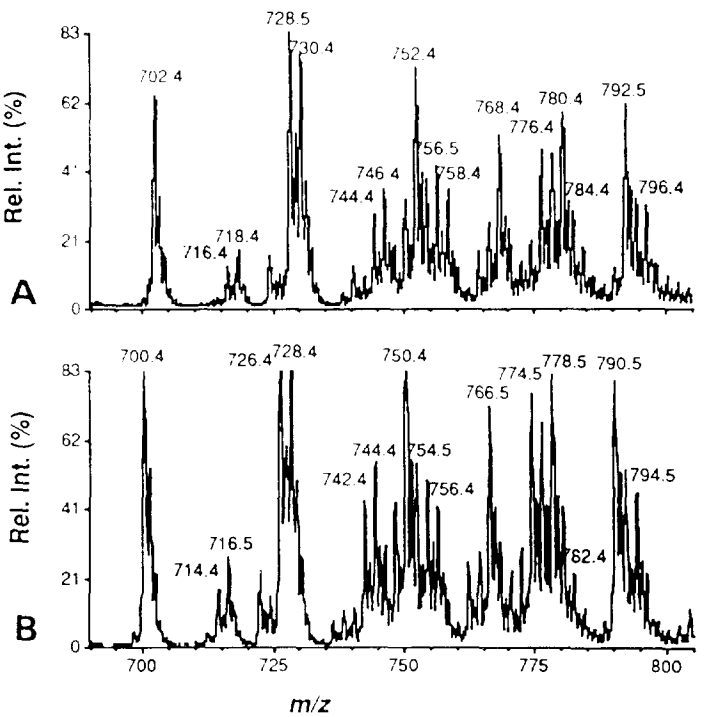

Fig. 11. Positive (A) and negative (B) electrospray spectra of bovine brain phosphatidylethanolamine. A solution of the sample (5 ng/ml) was pumped into the electrospray source at a flow-rate of 3 ml/min and the spectra were obtained on a QqQ-type instrument. Reproduced from Ref. [85] with permission.

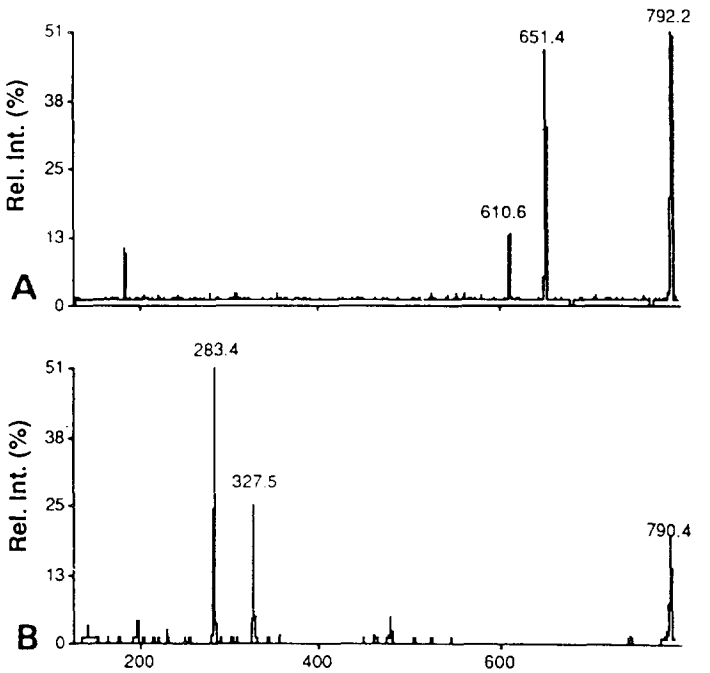

Fig. 12. Low-energy positive (A) and negative (B) ion CID spectra of the $[M+H]^+$ and $[M-H]^-$ parent ions from 18:0–22:6 diacylphosphatidylethanolamine. The spectra were obtained on a QqQ-type instrument using argon as the collision gas. Reproduced from Ref. [85] with permission.

the $[M-H]^-$ negative ions. CID in the middle quadrupole was used to assign the aliphatic chains and their regiospecificity. For example, the CID spectrum of the $[M-H]^-$ ion at nominal mass 776 showed carboxylate ions corresponding to 22:5 and 22:4 at m/z 329 and 331, respectively, as well as several lyso PE fragments. The species were thus identified as 18:0–22:5 and 18:1–22:4 alk-1-enylacyl GPE.

It is now possible to employ mass spectrometry to directly assess detailed events of phospholipid biosynthesis and metabolism through analysis of intact phospholipid molecular species [16]. Holbrook et al. [87] have used negative FAB-MS and FAB-MS–MS to study the molecular species of the GPL from phorbol ester- and bradykinin-stimulated PC12 cells. Except for phosphatidylethanol (PEt), the GPL were hydrolyzed to PA before being analyzed by MS. PEt was used as a marker for molecular species hydrolyzed by stimulated phospholipase D activity. Molecular species were determined from the $[M-H]^-$ ion in the spectra and linked scan analysis was used to identify the component fatty acids when more than one molecular species contributed to one $[M-H]^-$ ion.

Kayganich-Harrison and Murphy [88, 89] have described a method employing stable isotope labelling and FAB-MS–MS to directly assess events of biosynthesis and metabolism of arachidonic acid containing GPL molecular species by cells carried in culture. Mast cells were cultured with [$^{13}C_{17}$]linoleic acid in order to follow the conversion of this precursor into arachidonic acid which was then incorporated into cellular GPL. Use of the [^{13}C]linoleic acid precursor avoids the problem of changing the endogenous pool size by the direct addition of labelled arachidonic acid. Measurement of the [$^{13}C_{17}$]label also avoids interferences from endogenous isobaric fatty acids that are naturally present at low levels. The extent of label enrichment was monitored over a 24-h period in each arachidonate-containing GPL species by using negative FAB ionization

with selected reaction monitoring. The ratio of the [$^{13}C_{17}$]-labelled and unlabelled arachidonic was determined from the respective carboxylate anions at m/z 320 and 303, produced by CID from specific molecular species. Because the level of ^{13}C incorporation was less than 14% it was not possible to measure it by direct FAB-MS of the individual GPL classes. However, with precursor ion scans for arachidonic acid (m/z 303) and [$^{13}C_{17}$]arachidonic acid (m/z 320) it was possible to see the incorporation of [^{13}C]arachidonic in individual molecular species. The major labelled PE species were found to be 16:0p–20:4, 18:0a–20:4, 18:1p–20:4, 16:0a–20:4, and 18:1a–20:4. The isotope enrichment was even higher in the PC and PI fractions. Kayganich-Harrison and Murphy [89] found that the MS–MS method permitted measurements to be made at isotope enrichment levels as low as 0.005 to 0.01. They have also used this strategy to study the source of arachidonic acid that is metabolized to eicosanoids by 5-lipoxygenase [89].

The structure of GPL isolated from *Halobacterium halobium* has been studied by negative FAB and MS–MS of the $[M + H]^+$ species [90] and the major component of the polar lipids has been shown to be a methyl ester of PG phosphate.

Kloppel and Fredrickson [91] have described a FAB-MS–MS method for analysis of intact ether-linked polar ether lipids present at microgram levels in crude lipid extracts from halophilic archaebacterial cells (including *Halobacterium halobium*). The lipids were loaded onto a silica gel column and separated into three fractions by eluting with dichloromethane, acetone and methanol. The last two fractions (TGL and TPL) were analyzed by FAB-MS–MS. Negative-ion spectra showed the intact deprotonated lipid molecules and in some instances their deprotonated sodium salts and additional structural information of selected ions was obtained after CID. The most significant ion apart from the ion at m/z 612.2 in the mass spectrum of the TGL fraction was the ion at m/z 1217.7, which corresponded to the deprotonated sulfated triglycosylated analogue of 2,3-di-O-phytanyl-*sn*-glycerol. Confirmation of the presence of a sulfate group was obtained by the fragment at m/z 97 in the CID spectrum of the ion at m/z 1217.7. The negative mass spectrum of the TPL fraction contained a base peak at m/z 921.6 that corresponded to the deprotonated monosodium salt of the diphytanyl ether analogue of the methylated PG phosphate. CID of this ion produced cleavage of the ether bond between the glycerol backbone and the phosphate group, which after the loss of a water molecule results in a negatively charged fragment derived from the polar head group at m/z 269. In addition there was an ion at m/z 79 corresponding to the $[PO_3]^-$ group.

PAF (1-O-alkyl-2-O-acetyl-*sn*-glycerophosphocholine) is a mediator of inflammation and acute hypersensitivity reactions. Its identification in biological samples has been performed by MS–MS [92]; in particular, selected tandem mass spectra scans provided the improvement in signal-to-noise ratio necessary to obtain structure confirmation of PAF at the nanogram level in partially purified epidermal cell secretions of the salt-water catfish *Arius thalassimus*. A very sensitive FAB-MS–MS assay for PAF and acyl-PAF was based on the detection of the m/z 184 ion resulting from CID of the $[M + H]^+$ ion [93]. A detection limit in the low picogram range was claimed. Similarly a method based on reversed-phase HPLC combined with ionspray ionization and MS–MS has been reported [94]. By monitoring $[M + H]^+$ in SIM mode or the $[M + H]^+$ to m/z 184 reaction in MS–MS mode a detection limit of 0.3 ng was achieved for PAF. For lyso PAF the sensitivity was better in the SIM mode.

GPL, present in most higher organisms as minor constituents of the plasma membranes of cells, have been studied by FAB-MS–MS [95], while permethylated derivatives have been successfully characterized by DCI-MS–MS [96]. In both cases useful information of the ceramide structure was also gained by MS–MS.

Zhang et al. [97] have reported the analysis of intact HETE PCs using negative-ion LSI-MS–MS in a four-sector tandem mass spectrometer having a high-energy collision cell. Thus, they were able to determine structures of individual

HETEs and HETE PCs, while avoiding procedures such as enzymatic degradation and derivatization, that could lead to potential artifacts through autoxidation and degradation of the polyunsaturated fatty acids. Arachidonic acid is oxidized to regioisomeric 5(S)-, 12(S)- and 15(S)-HETE by the respective lipoxygenases and these hydroxylated fatty acids can be incorporated into cellular phospholipids. CID of the $[M-H]^-$ ion at m/z 319 from the HETE regioisomers produced ions, such as m/z 301 $[M-H-H_2O]^-$ and m/z 257 $[M-H-(H_2O+CO_2)]^-$. Ions characteristic of the particular HETE resulting from α-cleavages adjacent to the hydroxyl moieties were also formed. Negative LSI-MS of purified HETE-containing PC species gave three fragment ions appearing at m/z 810, m/z 765 and m/z 739 (Fig. 13a) corresponding to $[M-CH_3]^-$, $[M-HN(CH_3)_3]^-$ and $[M-CH=CHNH(CH_3)_3]^-$, respectively. There were also carboxylate ions at m/z 283 and m/z 319, corresponding to stearate at the *sn*-1-position and HETE at the *sn*-2-position, respectively. Similar fragmentation pathways have been observed in the negative FAB spectra of other eicosanoid-derived GPL. In order to characterize the HETE

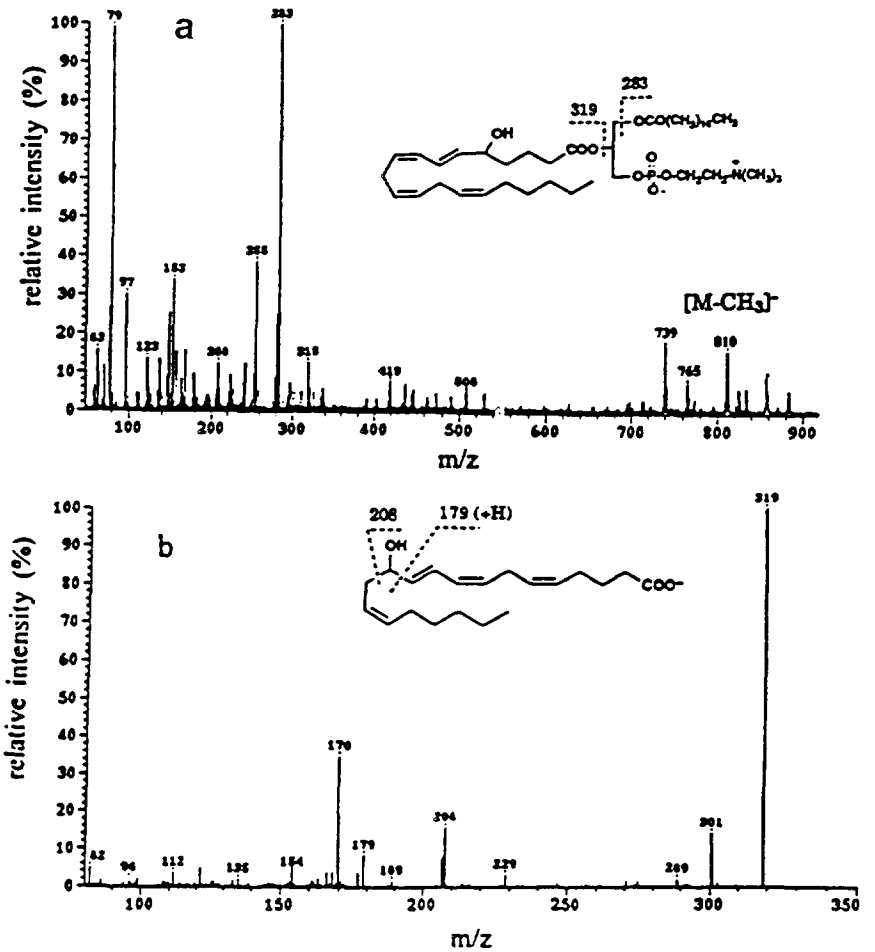

Fig. 13. The negative LSI mass spectrum of 12-HETE PC (a) and the high-energy CID spectrum of the m/z 319 parent ion from 12-HETE (b). The spectra were obtained on a four-sector EBEB tandem mass spectrometer equipped with an LSI ion source. The CID spectrum was obtained at a collision energy of 4 keV using argon as collision gas ($7 \cdot 10^{-3}$ Pa). Reproduced from Ref. [97] with permission.

moiety, the CID spectrum of the carboxylate ion at *m/z* 319 was examined and found to yield product ions similar to those observed using HETE standards. A comparison of the spectra with those of the standards clearly indicated the positions of the hydroxyl groups on the HETE molecules, thus allowing the HETE regioisomer present in the PC to be identified. CID of the *m/z* 319 ion resulted in the formation of product ions at *m/z* 115 and *m/z* 302 from 5-HETE PC, *m/z* 179 and *m/z* 208 from 12-HETE PC and a unique product ion at *m/z* 219 from 15-HETE PC. The CID spectrum for 12-HETE PC is shown in Fig. 13b. Zhang et al. [97] have provided some explanation for the formation of these fragments and have concluded that they are sufficiently reproducible and characteristic of the location of the hydroxyl groups in the HETE PCs to allow the confirmation of the structures of individual HETEs present in each of the PCs. The CID spectrum of the $[M - CH_3]^-$ ion from the HETE PC showed abundances for the carboxylate ions at *m/z* 283 and *m/z* 319 ions in the ratio of 4:1. This was another exception to the rule that the ion from the *sn*-2 position is more abundant than that from the *sn*-1 position.

Morrow et al. [98] have reported the discovery of biologically active D_2/E_2-isoprostanes that are formed in vivo as a result of nonenzymatic free radical-catalyzed lipid peroxidation. MS and MS–MS was used to show that these isoprostanes are formed in situ esterified to GPL. A normal-phase HPLC fraction containing D_2/E_2-isoprostanes was first found to elute in a fraction running later than normal PC. The negative LSI-MS spectrum of the polar lipid fraction had ions at *m/z* 843 and 798 corresponding to the masses of the M-15 and M-60 fragments of PC containing stearate and D_2/E_2-isoprostane. In addition the spectrum contained carboxylate ions of D_2/E_2-isoprostane and stearate at *m/z* 351 and 283, respectively. High-energy CID of the *m/z* 351 ion produced ions at *m/z* 333, 315, and 279 that are also fragments in the spectra of authentic PGD_2 and PGE_2.

4.2.3. Ceramides and sphingomyelins (SPH)

Ceramides, which are amides of long-chain bases such as sphingenine with fatty acids, constitute the lipophilic tail of GPL and SPH, and are also present in some animal waxy secretions. These molecules, either naturally occurring as such or deriving from the catabolism of sphingolipids, recently gave rise to much interest, because of their postulated role as modulators of cell growth and differentiation [99,100]. FAB-MS–MS analysis of the molecular species of ceramides [101–103] and SPH [85,104] has been already reported and reviewed [5]. Underivatized ceramides and SPH show poor sensitivity by FAB-MS. Costello and Vath [101] have shown that reduction of the amide to an amine leads to considerable improvement in sensitivity (1000-fold for ceramides). Furthermore, reduction with borane yields an alkylborane at all double bonds, which can be oxidized to a secondary alcohol with H_2O_2. Costello and Vath [101] also demonstrated that CID of the $[M + H]^+$ ions of the derivatized ceramides provided additional information about the mass of the N-acyl chain. In addition, CID of ceramides derivatized by hydroboration and H_2O_2 oxidation could reveal double-bond location [105]. Instead of chemical derivatization of ceramides, Ann and Adams [102,103] found that the lithium adduct, $[M + Li]^+$, greatly improved the information content of the CID spectra by yielding diagnostic fragment ions that provide more extensive information about the structures of the sphingoid and N-acyl chains. In particular, the CID spectrum of N-palmitoyl-(4E)-sphingenine yielded an abundant series of high-mass product ions formed by charge-remote closed-shell product ions, which are 14 u apart for a saturated alkyl chain and end with the K ion, that immediately revealed that the N-acyl chain is derived from palmitic acid. As in the case of fatty acids charge-remote fragmentation [106] of ceramides can also reveal the location of double bonds and hydroxyl groups on the N-acyl chain. Structures of individual components of mixtures of ceramides can also be directly determined by CID of the alkali metal ion adducts, and the detection limit for structural determination is 8 pmol [102].

Rubino et al. [104] have used a FAB-MS–MS procedure involving precursor and fragment ion analysis to determine the fatty acid and long-chain base composition of a complex ceramide

mixture prepared from beef brain lipids. Although most spectra were made on a BE-type sector mass spectrometer equipped with a FAB source and a collision cell, some measurements were also made on a QqQ-type mass spectrometer. They concluded that the two instruments yielded complementary results and both were necessary for the full characterization of the compounds. The main advantage of the QqQ instrument is the higher mass resolution available for precursor scans that allows for the discrimination of species that have the same number of carbon atoms but different degrees of unsaturation [107]. Otherwise similar results were obtained with both instruments. The most abundant ceramide molecular species detected in the source spectra were structurally characterized by high-energy CID fragment spectra of the $[MH - H_2O]^+$, $[M + Li]^+$ and $[M + Na]^+$ ions. The spectral features were very similar to those reported by Ann and Adams [102,103,108]. The characterization of the long-chain bases was attempted by fragment ion analysis of the signals at *m/z* 360, 282 and 264. The CID spectrum of *m/z* 264 yielded scarcely any fragmentation, while the CID spectrum of *m/z* 282 was very similar to that of authentic C_{18} sphingenine. The adverse result from *m/z* 264 was not surprising, since no charge-remote fragment array is found starting from *m/z* 264 in the CID fragment ion spectrum of *m/z* 284 from sphingenine [109]. The fatty acid structure was determined by CID fragment analysis of the appropriate $[M + Li]^+$ ion. Thus it was possible to locate the double bond of nervonic acid (24:1) at carbon 15. Rubino and Zecca [109] compared the ceramide fatty acid composition determined by FAB-MS and FAB-MS–MS with that determined by GC–FID and GC–EI-MS. Since the ceramide mixture used contained only one LCB, the fatty acids could be determined from the $[MH - H_2O]^+$ and $[M + Li]^+$ profiles as well as from precursor ion analysis. All the compositions derived by MS were rather similar, but MS results differed from the GC results. For example, palmitic acid was not detected by FAB although it was found to be 10% of the total by GC–MS. Some discrepancy between the values obtained with the two techniques can be attributed to the very different surface activity of the short- and long-chain fatty acids.

SPH are prominent structural components of natural biomembranes. The molecular species of SPH are of interest in elucidating specific physiological functions, especially in cellular membranes (lipid bilayers), but also in lipoproteins and other sources rich in lipids. Analysis of molecular species of SPH has been performed in a variety of ways including on-line LC–MS with discharge-assisted TS mass spectrometry [110,107].

MS–MS has recently been used by Ann and Adams [103] to study the structures of SPH. They used an EB sector instrument with a FAB source and a collision cell in the first field-free region between the source and the electric sector. In the positive FAB mode 3-nitrobenzyl alcohol (NBA) or NBA plus a cation modifier was used as a FAB matrix to generate $[M + H]^+$ or $[M + Cat]^+$, where Cat = Li^+, Na^+, K^+, Rb^+, or Cs^+. In the negative FAB mode, an anion triplet corresponding to $[M - 15]^-$, $[M - 60]^-$ and $[M - 86]^-$ was generated using triethanolamine as the FAB matrix. CID of the $[M + H]^+$ ion produced only the *m/z* 184 ion which indicated the phosphorylcholine group. Alternately $[M + Cat]^+$ ions produced ions giving information about the nature of the intact ceramide and also about the nature of the N-acyl chain but still no structurally definitive product ions. The negative triplet on the other hand yielded high-mass charge-remote product ions arising mainly from the N-acyl chain and ending at the peak labelled K. These ions yield information about the location of double bonds and other substituents. In the case of N-oleoyl-(4E)-sphingenine there is a 54-u gap in the spectra due to unfavourable vinylic cleavages to and through the double bond. This type of information, which is not available from the same anion triplets of PC, is thought to be due to the inherent stability of the amide bond [103]. Fig. 14 shows the CID spectra of two $[M - 15]^-$ ions (*m/z* 797 and 715) in the negative FAB-MS of the SPH of bovine brain. The K and O ions at *m/z* 503 and 449, respectively, indicate that the

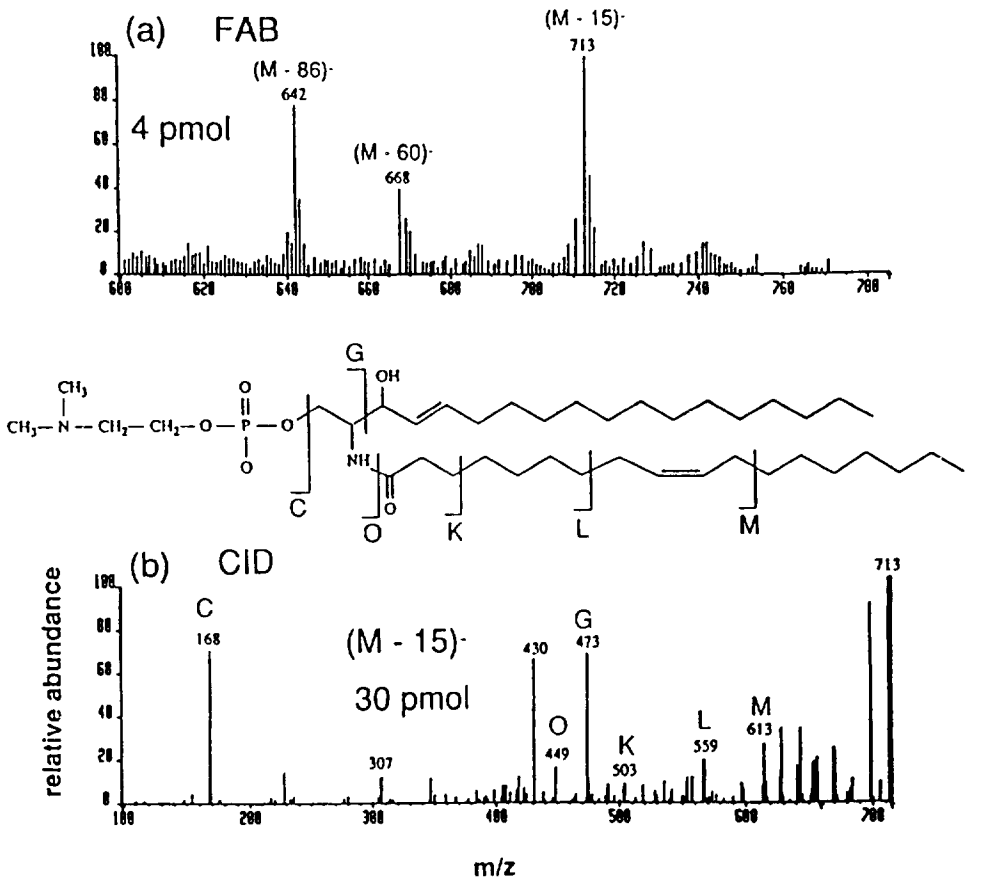

Fig. 14. The negative-ion FAB spectrum of 4 pmol N-oleoyl-(4E)-sphingenine phosphorylcholine (a) and the high-energy CID spectrum of the [M − 15] parent ion from 30 pmol of N-oleoyl-(4E)-sphingenine phosphorylcholine (b). The spectra were obtained on an EB-type sector mass spectrometer equipped with a FAB ion source. The CID spectrum (constant B/E scan) was obtained at a collision energy of 8 keV using helium as collision gas (50% beam transmission). Reproduced from Ref. [103] with permission.

sphingoid base is sphingenine. The spectrum in Fig. 14b shows ions resulting from cleavage at L and M indicating a double bond in the acyl chain. The 54-u gap between the masses of the L and M ions indicate that the SPH is N-nervonyl-sphingenine phosphorylcholine. In contrast, the lack of such ions in the spectrum of Fig. 14a indicates that the SPH is N-stearoyl-sphingenine phosphorylcholine. For complex mixtures, Ann and Adams [103] claimed a CID detection limit of 30 pmol.

Hayashi et al. [111] studied phosphonosphingolipids by FAB-MS and CID-MS–MS. The positive- and negative-ion FAB mass spectra of the phosphonosphingolipids of abalone show abundant $[M+H]^+$ and $[M-H]^-$ ions and although the FAB spectra were complex, the problems were overcome by the use of CID-MS–MS which provides excellent results for structural elucidation. In the high-mass region, charge-remote product ions, which may arise from cleavages of the N-acyl chain, were observed.

Kerwin et al. [85] have shown that positive- and negative-ion ES-MS and ES-MS–MS is a rapid and sensitive method for structure determination of molecular species of intact SPH. The commercial preparations of SPH analyzed yielded positive- and negative-ion ES spectra for

molecular species, which were similar to those described above for the GPL. Bovine SPH yielded 17 molecular species. Attempts to increase fragmentation to generate peaks characteristic of the N-acyl and sphingenine/sphinganine constituents using 1% methanolic formate were not successful.

4.2.4. Other derivatives

Coenzyme A thioesters of long-chain fatty acids are essential intermediates in the biosynthesis of fatty acids and glycerolipids. In the past analysis of intact acyl CoAs has involved direct and continuous-flow positive mode FAB-MS [112–114]. The spectra showed a base peak at m/z 136 due to protonated adenine along with the $[M + H]^+$ ion and fragment ions indicative of the CoA and acyl group.

The utility of this approach was demonstrated in a recent report where tandem mass spectrometry of positive ions from 3-keto-2-propylpentanoyl-CoA was used to probe detailed structural features in this unique CoA thioester [115]. Zirolli et al. [116] have investigated the negative-ion FAB-MS and tandem mass spectrometry behaviour of long-chain acyl CoAs. The negative-ion FAB spectra of long-chain acyl CoA esters are characterized by the formation of an abundant $[M - H]^-$ ion and two types of fragment ions, one prominent set which retained the acyl group (I–V) and another less abundant set (A–E) which was related to phosphopantethene and adenine portions of the CoA. The ions, which retained the acyl group in the spectrum of palmitoyl CoA appeared at m/z 675 (I), 657 (II), 595 (III) and 577 (IV). The ion at m/z 675 is a result of fragmentation at the 5′-phosphate ester bond and corresponds to the acyl diphosphopantetheine group. The m/z 595 ion is formed by cleavage at the central oxygen of the diphospho ester bond and corresponds to the acyl monophosphopantethene group. Loss of H_2O from these two ions leads to the ions at m/z 657 and 577, which in turn decompose by loss of alkylketene to form the ions at m/z 357 (D) and 339 (E).

In contrast to the direct FAB spectrum, the CID spectrum of the $[M - H]^-$ ion from palmitoyl CoA (m/z 1004), showed abundant ions for the adenine-containing ions and weak or missing acyl-containing fragments. These results suggest that the formation of many characteristic ions observed in direct FAB analysis occurred during the desorption process. CID and a precursor scan of the m/z 339 ion can be used to reveal the CoA species present in complex acyl CoA mixtures. Alternately individual species can be identified by neutral loss scans of the mass of particular alkylketenes. These principles are illustrated in Fig. 15 which shows the negative-ion FAB-MS and FAB-MS–MS spectra of a mixture of standard acyl CoA thioesters. The total FAB spectrum in Fig. 15a shows the deprotonated molecular ions as well as several series of abundant fragment ions. The acyl chains can be determined from the precursor ions of the ion at m/z 339 (Fig. 15b). Fig. 15c shows the precursor ions resulting from the neutral loss of hexadecylketene.

5. Conclusions and future prospects

During the last few years MS–MS has developed from an experimental curiosity into an analytical routine. Until recently fast atom bombardment has been the method of choice but it is presently being replaced by electrospray ionization. Originally intended for high molecular mass solutes, electrospray is equally well suited for the ionization of the polyfunctional fatty acids and their glyceryl and phosphoglyceryl esters. Structural details are precisely determined for components of complex mixtures even when present in minute amounts. Although some of the need for chromatography is eliminated by MS–MS, the addition of on-line chromatographic separation promises to increase the usefulness of the technique for work with natural lipid mixtures. These systems are capable of handling total lipid extracts of progressively lower concentration and more complex nature. Both could accommodate further automation of lipid class and molecular species analysis by combining clean-up, derivatization (if necessary) and simplified chromatography. The introduction of electrospray ioniza-

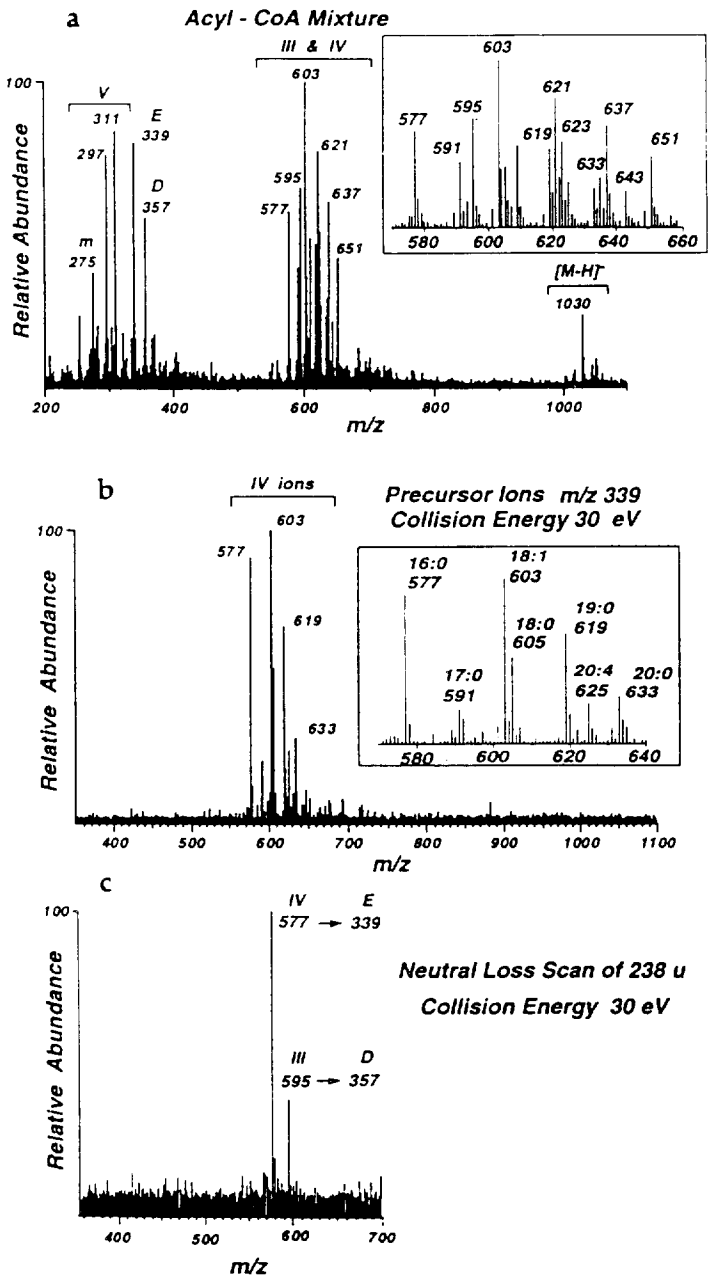

Fig. 15. FAB-MS of a mixture of acyl CoA's containing $C_{16:0}$, $C_{17:0}$, $C_{18:0}$, $C_{19:0}$, $C_{20:0}$ and $C_{20:4}$ acyl species (a). The spectrum was obtained from a glycerol matrix and the letter m designates matrix ions; precursor ions of m/z 339 obtained following CID of source formed ions, m/z 350–1100 from the same mixture of acylCoA species (b); and precursor ions of the neutral loss of hexyldecylketene, 238 u, obtained following CID of source formed ions, m/z 300–700, from the same mixture of acyl CoA species (c). Spectra were obtained on QqQ-type instrument equipped with a FAB ion source and CID were obtained at 30 eV collision energy using argon as collision gas (67 Pa). Reproduced from Ref. [116] with permission.

tion has permitted direct quantitation of the more polar lipid species.

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